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Mycotoxins in South-East Asia?

The US government says that chemical weapons have been used in Indo-China, but its evidence falls short of proof. Even so, there is a case to be answered. How?

Last week's account by the State Department of chemical warfare in South-East Asia is a little like the discovery of quasars: something is undoubtedly going on, but it is impossible to tell just what. Part of the difficulty is that there is so little objective evidence. The principal allegation (see *Nature*, last week) is that the Vietnamese have been using chemical weapons of some kind in the wars in which they have been or are still engaged in Laos and Kampuchea (Cambodia), that the chemicals concerned include toxins of the kind produced during the growth of fungi of various *Fusarium* species and that these have been manufactured in the Soviet Union. The allegation, already vigorously denounced as "propaganda" by the Soviet news agency TASS, was first made public by Mr Alexander Haig in Berlin last September, but was at the time almost entirely unsubstantiated. Since then, scraps of information have been squeezed like blood from a stone from the State Department. Last October, the State Department gave the *ad hoc* group of experts set up to investigate the charges by the United Nations the results of the chemical analysis of samples of vegetation and other materials said to have been collected from Laos and Kampuchea. Now, it seems, there is more to say. The State Department clearly thinks it has evidence with which to prove its case. But only four samples of supposedly contaminated material have so far been analysed in the laboratory. The circumstances are exactly analogous to those in the early days of the quasars, when the implications of a meagre stock of data were clearly so huge that people could not summon up the fortitude to follow the prudent course — to wait for more data to accumulate. What, in these circumstances, is to be made of the State Department's report?

Although there are some numbers to play with, anecdotal evidence remains the most compelling part of the State Department's case. Reports of interviews with refugees from Laos and Kampuchea now in camps in Thailand have several strikingly common themes. Over a period of five years, they say, villages in central Laos and on the Kampuchean border with Thailand have been attacked with sprays, bombs or rockets releasing toxic agents. The heads of the rockets are said to have released red, blue or white smoke, while the sprays are now widely known as the causes of "yellow rain". The reports of the damage done to people are too many to be overlooked — or to have been fabricated. Nausea, dizziness and respiratory and circulatory troubles are the milder symptoms. Bleeding gums and other haemorrhagic phenomena are common. Statements that the corpses of those who have died are often bloated, and that their digestive tracts have rapidly become bloated, are a common theme in the reports of these first-hand accounts of attack from the air. And the State Department's star witness, a Vietnamese pilot who defected in 1979, has given such a specific account of how he carried out air attacks with unusual and presumably toxic

weapons that those wishing to declare it false could fairly be asked to provide a specific and not a general denial.

Like all anecdotal evidence, of course, accounts such as these, however gripping, do not constitute proof. Interviews conducted by intelligence officers in far-away refugee camps do not have the objectivity even of the kinds of conclusions that emerge from examinations carried out by lawyers in courts of law. Indeed, in South-East Asia, the process of gathering verbal evidence must have been made even less certain by the difficulty encountered by the United Nations expert group on its flying visit to Thailand at the end of last year — the need to translate from a local dialect into Thai and from that into English. From the evidence now published, there is no way of telling how far witnesses may have been led to a desired tale by the questions asked of them, no simple way of making sense of statements that do not ring true (such as the assertion that yellow rain was sprayed from a height of 7,000 feet) and no simple way of being sure that the time-course of the conditions caused by the supposedly toxic chemicals were accurately described. The weight of the verbal evidence that toxic weapons have been used in South-East Asia therefore rests on the amount of it, not on its quality.

Because of such doubts, objective scientific evidence is potentially crucial. Here the difficulty is that there is far too little of it. Only four samples of material have so far been analysed for trichothecenes, the toxins produced by *Fusarium* species. According to the laboratory of Dr Chester J. Mirocha of the University of Minnesota, where the analyses have been carried out, the figures given in the State Department's report represent the proportions of trichothecenes in the whole bulk of the samples which have been analysed, so that if a leaf or water has been contaminated by some artificial external agency, the concentration of the toxins in that source must be higher, probably very much higher, than the concentrations that have been reported. In any case, the nub of that part of the State Department's case that rests on the data now made public, is that the concentrations now reported are greater than those found in grain that has been contaminated with *Fusarium* fungi in natural circumstances. The comparison seems to be generally accepted. Even allowing for the improved methods of analysis now available, it is unlikely that the samples recovered from South-East Asia have been contaminated by the natural growth of *Fusarium* species on the spot. But even this is not the clinching argument the United States State Department would like it to be.

The several objections to the use of these data will seem pettifogging to those who know what case they want to prove, but are nonetheless worth stating openly. First, four samples of such small bulk and of such different kinds are hardly enough to demonstrate the internal reproducibility of the results now arrived at. Worse still, those responsible for the chemical analysis have no knowledge of how the samples they have dealt with were collected, stored and transported. Indeed, they can have no way of being sure that the mycotoxins whose presence they have demonstrated were not added to the samples by some agency or individual anxious that the State Department should have a persuasive tale to tell. This dark suspicion, which may seem unjust to a responsible government such as that of the United States, cannot unfortunately be shaken off, for the intelligence community responsible for collecting the samples has a shaky

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reputation for veracity. The only solution to this dilemma is, of course, that those responsible should acknowledge that this potentially objective part of their case will be convincing only when there is much more material for analysis whose authenticity can be demonstrated.

For the time being, the analytical evidence falls far short of being compelling, at least within the professional community. The point can be simply put by means of the conventions of the scientific literature. Thus the State Department has agreed that Dr Mirocha and his colleagues may, if they wish, publish the results of their analyses in the scientific literature. (So, of course, they should do.) The abstract of an acceptable contribution to the literature along these lines might take the form:

We have analysed four samples of materials supplied by the State Department and have obtained the following results. . .

Referees would no doubt say that such a report was technically beyond reproach but of somewhat limited interest. But if the proposed publication has an abstract beginning:

We have analysed samples collected from four sites in South-East Asia soon after the use of chemical weapons . . . and have concluded that the Vietnamese have been using toxic agents derived from *Fusarium* species . . .

the referees would be down on them like a ton of bricks. This does not imply that the State Department's case falls down but rather that even with the apparently objective evidence for it now added, the case remains largely circumstantial.

The State Department has not yet proved its case, but it has raised a question that cannot be ignored. It is in everybody's interest that it should be answered, and quickly. One of the most chilling passages in the State Department's report is the speculation — it is no more — that mycotoxins rather than more lethal agents have been used in South-East Asia because they lead to bizarre forms of death calculated to frighten away the survivors of attacks. (Certainly the refugee camps of Thailand have been full for months.) If indeed it has now been shown that chemical weapons like these do have some military value, the outlook is awesome not merely for the luckless people of Laos and Kampuchea but for the rest of us. The prospect that it might be possible to reach an agreement between the major military powers to outlaw the use of chemical weapons in future wars, and to abandon their manufacture, will clearly be diminished if some group of generals has it firmly in its collective head that such a treaty would be a military loss.

So what should be done? The United Nations group of experts should clearly be asked — and helped — to complete the study on which it has embarked. The practical need is that it should be able to make a more detailed investigation of the regions in South-East Asia in which mycotoxins are said to have been used. The Soviet Union, which has some influence with the government of Vietnam, should recognize that its own case — that the allegations are a pack of lies — could only be strengthened by an investigation on the spot. And the United States should understand that the glee with which it has accused the Soviets of complicity in whatever has been happening in South-East Asia is counterproductive. Thus it serves no purpose but to confuse, that the State Department's report says that techniques for manufacturing and spraying trichothecenes from the air have been developed in the Soviet Union without adding that the objective (fully described in the literature) has been to control the spread of fungi other than *Fusarium* and related genera in forests. One of the materials, the trichothecene T-2, has even been canvassed as a rodenticide. (Nobody suggests that it would make sense to think of killing rats by spraying such a material from the air.)

What the State Department has not appreciated is that the question which it has legitimately asked will be regarded less seriously by those who alone can provide an objective answer if the statement of the problem is biased. The danger that the appearance of the State Department's document will be mistaken for a political ploy is in any case enhanced because the United States government is in the thick of persuading Congress to embark on the manufacture of binary chemical weapons in the

form of the 155 mm artillery shell known as M687, rightly castigated last week by Dr Matthew Meselson of Harvard University (before a Senate subcommittee) as militarily pointless and politically inflammatory. At the same time, to be fair, President Reagan has asked that the United States delegation to the Committee on Disarmament in Geneva reopen discussions on measures to control chemical weapons. The zeal with which those talks are prosecuted will be a powerful determinant of people's willingness to answer the State Department's question.

Professional propaganda

By what means should professional people, not only scientists, aim to influence public opinion?

The professions have often been accused of indifference to the problems of the societies in which they are embodied, and there is substance in the charge. Now, when many industrialized states are faced with unfamiliar and serious economic crises, governments may often rightly grumble that academics are more concerned with their own skins than with the solution of the economic problems about them. Other professions are similarly indictable. During the housing boom of the 1960s, for example, architects built dwellings without recognizing that people might not want to live in them, while teachers have for decades taught in schools without paying sufficient attention to the fitness of the curriculum for their students' needs. Not all, however, is disappointment. In many places, the legal professions have taken the lead in linking the practice of the law more closely with social realities and needs, while physicians have done much to change people's attitudes towards health and disease. So is it not entirely to be welcomed that there has recently been a rash of professional groups taking a special interest in what may be the most serious of all contemporary social problems — the dangers of nuclear war?

The short answer, which is "no", appears to offend some of those concerned (see page 386). That professional people have a responsibility to contribute their special knowledge to the solution of general problems is not in dispute, but only the means by which they do so. Similarly, there is no question that when a profession is, broadly speaking, agreed about the social consequences of some circumstance lying largely within its field, it should declare itself. It would thus be shocking if physicians were silent about the probable consequences of smoking. Unfortunately, however, the dangers of nuclear war do not lie exclusively within the province of a single profession, while there can be very few professions that are in broad agreement within themselves about the steps to be taken to head off the danger.

This is why the emergence of groups of architects, or teachers, or even scientists, with catchy acronymic titles "against nuclear war", is a misfortune. By suggesting that the whole of a profession is in some way united about what should be done, these groups give the general public a false impression. By being separate from the more broadly based groups of people with the same aims, they run the risk of over-simplifying important problems. Medical groups, for example, may fairly make fun of official plans for civil defence against nuclear attack, but it would be more constructive if they did so in the context of the appalling difficulties, political and otherwise, of avoiding dependence on nuclear weapons.

In Britain and elsewhere in Western Europe, the immediate question is not whether nuclear war would be devastating — everybody is agreed on that — but whether individual governments should to some degree opt out of present arrangements for the defence of Europe. Another question is the basis on which arms control agreements might be negotiated. Professional groups usually profess no opinion on such practical questions, preferring to stay within the confines of their professional competence. The result is that, by what they say, they influence general opinion irrationally or at least subliminally. This is why they are open to the charge of deceit.

Industry funds in universities

New guidelines emerge from Pajaro Dunes

Washington

Universities should only accept research funds from private companies if secrecy is kept to the absolute minimum necessary for patent protection. At the same time, companies which fund such research should normally be entitled to receive exclusive licences on any useful results that emerge, at least for a period sufficient to prevent rival companies from unfairly exploiting the same research.

These were two of the principal conclusions to emerge from a three-day meeting between the presidents and selected faculty members of five top US research universities, and senior executive officers of ten leading biotechnology companies, held at the California coastal resort Pajaro Dunes at the end of last week.

The conference had been organized largely at the suggestion of Dr Donald Kennedy, president of Stanford University. Its main purpose was to discuss a range of controversial issues that have emerged over the past few years as universities have looked to industry as an alternative source of research support from the federal government and have increased their efforts to push research results into the market-place. Companies in turn have been turning their attention to the frontiers of biomedical research in their quest for new and improved products and industrial processes.

Others attending the conference included the presidents of Harvard, Massachusetts Institute of Technology, University of California and California Institute of Technology. Each had been asked to invite one university administrator, two faculty members with direct or indirect experience of dealing with outside companies, and two senior executives from companies with experience of sponsoring university research. The companies represented at the meeting included Beckman Instruments, Syntex, Cetus Corporation, Applied Biosystems Inc., Gillette Corporation, Eli Lilly, DuPont and Genentech.

Dr Kennedy stressed after the meeting that the purpose had not been to agree on rigid rules that should apply to each university, but rather to work out what he described as a "framework for future relationships between universities and industry". Faculty members at several of the universities represented at Pajaro Dunes had already indicated their concern that it should be left to each university to

decide how broad principles should be interpreted into policy — a policy that last week's meeting was quick to endorse.

Nevertheless, criticism that the conference had been limited to senior administrators and scientists on both sides was expressed in a letter to the participants signed by 25 scientists at research universities across the country, as well as several prominent union and consumer spokesmen such as Anthony Mazzochi of the Oil Chemical and Atomic Workers, and Ralph Nader.

The letter invited the participants to attend a second conference later this summer, at which the same topics will be discussed but primarily from the point of view of groups both within and outside universities which felt they had been unfairly excluded from last week's meeting. The letter quoted from the final speech delivered by President Eisenhower, in which he criticized not only the growing power of the "military-industrial complex" but also the danger that important policy issues were increasingly

being decided by a scientific and technical elite, rather than through open democratic processes.

Dr Kennedy has already agreed to take part in this second conference. He also said on Saturday that he would help the organizers of the conference to raise the necessary funds.

The Pajaro Dunes meeting produced agreement on an 11-page statement which set out some principles as a basis from which individual universities can develop guidelines and codes of conduct. The statement stressed, for example, that although links should be encouraged between faculty members and outside companies since these were considered mutually beneficial, "professional relationships with commercial firms should not be allowed to interfere with responsibilities for teaching and research".

The statement also said that in general it was not appropriate for universities to own substantial equity in companies which were staffed by their own faculty members. This is a sensitive point at Harvard, which

Flying start towards French law

The proposed French "research law", on which many of the plans of the minister for research and technology, Jean-Pierre Chevènement, depend, has cleared its first hurdle with a flying leap. The Economic and Social Council — a kind of litmus paper of the French nation — was not satisfied with giving mere approval to the law last week. Rather, it rearranged and added sentences to the draft bill to stress the significance of the new plans for France, indicating that they must be considered to be political priorities.

This is no slight thing, as the finance minister, Jacques Delors, recently put all spending ministries (including Chevènement's) on a tight rein. Something like a quarter of the new money offered to laboratories by Chevènement has had to be frozen, despite the minister's strong opposition. But, says the Economic and Social Council, "expenditure on research and development must escape, so far as is possible, from the present economic difficulties".

Money was not the only thing the council had in mind. Its members considered that the links between research and education need to be tightened; and so the council added phrases to the law which imply that Chevènement and the minister for national education, M. Alain Savary, must get together quickly to work something out, before Savary presents his own law to parliament in the autumn.

The council also stressed regionalization, sharpening the definition of the proposed "regional consultative committees" on research and technology.

It suggested words guaranteeing the mobility of personnel, and, in particular, emphasized the use of the French language in science. The use of French is "a fundamental objective" said the council, but admitted that it would be difficult to achieve. The research law should define a precise strategy, and the ministry should keep the council informed of progress on this issue, the council requested.

The broad support of the council for the research law will be welcome and significant, because the council is an important — if unusual — constitutional body. Its 200 members are drawn from all walks of life, but particularly from the working classes (as 140 members are supported by trades unions); and it gives its opinion and advice on such matters as are referred to it by the prime minister, or on matters that it chooses to study on its own behalf. Its advice is usually taken before major bills are put before the National Assembly, and although the advice is not binding, it would be politically inept to ignore it. Moreover, since the unions are strongly represented on the council, and since the same unions have a strong influence on the present socialist government, the council might be thought to have more weight than it had in the past.

The next ports of call for the draft law will be the Council of State (to check legality), the Council of Ministers (for final political approval by the government), and ultimately — perhaps by early summer — the National Assembly for parliamentary debate, and, if successful, passage into law.

Robert Walgate

eighteen months ago backed away in the face of a flood of national publicity from a proposal that it should share the equity in a company being set up by members of its department of biology.

Both university and industry representatives agreed that research agreements should require the minimum amount of secrecy, and that in general university scientists working with industry funds should retain full rights of disclosure on their research results.

One controversial topic which was discussed at the meeting was how to avoid potential conflicts of interest when a university scientist is involved with both university and private research teams working in closely related fields. The participants agreed that universities should be encouraged to draw up explicit conflict-of-interest rules, and also that any research agreements with private companies should not "impair the education of students, interfere with the choice by faculty members of the scientific questions or line of inquiry they pursue, or divert the energies of faculty members from their primary obligations of teaching and research".

There was apparently more disagreement on whether it was appropriate to grant companies which sponsor university research an exclusive right to use the results of that research. Some participants argued that this was going too far, and that although the company should be offered a royalty-free licence, the patent should also be offered to others at the same time. Some argued, however, that non-exclusive licences would discourage companies from developing a new product, and that an exclusive licence to the results was an appropriate *quid pro quo* for research support. If exclusive licences were not permitted, companies might be reluctant to support university research, said President Derek Bok of Harvard University.

The meeting had been described as "Asilomar II", a reference to the 1975 meeting on the safety of recombinant DNA research which took place a few miles away from Pajaro Dunes. Just as the earlier meeting had provided the basis for the subsequent development of the safety guidelines on DNA research, Dr Kennedy said after last week's meeting that it had marked the beginning of an attempt to establish a national consensus on guidelines for collaboration between universities and industry.

News of the new guidelines, however, is likely to evoke just as much controversy as the original Asilomar document. Graduate students at Stanford have already organized a series of meetings to discuss their perspective on the impact of the new university/industry links (*Nature* 25 March, p. 283). The Pajaro Dunes meeting had defined the arena in which future debates between supporters and critics of close university/industry links will inevitably take place.

David Dickson

Polish science

Travelling again

A few Polish scientists are now arriving in Western laboratories and universities to take up exchange places arranged before the imposition of martial law. The delay in their arrival has not been due simply to the general confusion following the army takeover; they bring with them news of emergency regulations for scientific trips, which demand, in particular, that any such trip must be "closely in line with the aims of the foreign policy of the Polish People's Republic and the socio-economic and scientific policy of the country".

Under these emergency regulations, any proposed trip must be submitted for detailed analysis by the director of the institute or rector of the university where the applicant works. This has to take into account not merely the scientific purpose and the cost-effectiveness of the trip (especially as regards foreign currency), but also whether or not the applicant can be guaranteed "to represent the political interests of the Polish People's Republic". In particular, trips will not be authorized for persons "who have actively worked to the detriment of the state, or who have broken the regulations of the decree on martial law". (This clause, if strictly applied, would exclude scientists working in several institutes of the Academy of Sciences, the Swierk nuclear research institute, and other academic establishments which responded to the imposition of martial law by protest strikes.) Relatives of scientists already working abroad will not be permitted to join them, although it is not made clear whether this is simply due to the shortage of currency, or whether it is intended as a means of ensuring that the scientists concerned will return at the end of their tour of duty.

Preferential treatment, it seems, will be given to long-term exchanges, especially if they are concerned with research into subjects of particular significance for the Polish economy, and if the proposed visit is to a "leading" scientific institution abroad where the scientific and practical reward is self-evident. Visits planned under existing exchange agreements and contracts for visiting lecturers will also be given a more favoured status, while the officials of international scientific societies, and academics invited to take the chair at international congresses and symposia (if not otherwise disqualified) should be enabled to travel "in order to facilitate the participation of Polish scientists in leading international scientific events".

Students, on the other hand, are to have their applications "delayed", except for students studying at universities in socialist countries on the basis of international agreements, and students sent abroad by the Ministry of Science, Higher Education

and Technology for one- or two-semester "short courses". The same delaying process is to be applied to researchers wishing to go abroad to collect archive material, consult with their colleagues on "non-priority" matters, or to take a "passive part" in an international conference.

Vera Rich

Universities in industry

Imperial goals

Imperial College in the University of London has set up a manufacturing company with venture capital. The college, which is providing the facilities for the new company, will share the equity equally with Technical Development Capital Ltd (TDC), a subsidiary of the Finance for Industry Group, which is putting up £400,000 as initial investment. Imperial Biotechnology Ltd, as the new company is called, will use the college's pilot fermentation plant to manufacture purified enzymes, proteins and specialized fermentation products. These will be used mainly for the chemical and pharmaceutical industries.

The new company is primarily intended to provide a solution to the college's recent difficulties in funding the fermentation plant, which is housed in the biochemistry department. Although the plant had been earning contract money, the college could no longer make up the running costs out of central funds. It is hoped that the new company will be at least self-financing, eventually achieving a turnover of a few million pounds per year.

About twenty staff working with the fermentation plant will be transferred from the college payroll to that of the new company. They will retain their existing commitments to teaching and research. Management of the plant, however, will be transferred from Professor Brian Hartley, head of the biochemistry department, to a new management team appointed by TDC. Dr Trevor Langley, formerly with Whatman Biochemicals Ltd., has been made managing director of Imperial Biotechnology Ltd.

Professor Hartley, a critic of the government's unwillingness to support more biotechnology posts in British universities, plans to use some of the money released by transferring staff to the company payroll to establish two new academic posts in the Centre for Biotechnology which he is at present establishing.

Imperial Biotechnology Ltd will operate much as any other company, using profits to finance expansion. It will, however, benefit from its position on the college campus by maintaining close links with research. Professor Hartley, who has links with the Swiss company Biogen, will also be scientific adviser to the new company. He sees no conflict of interest.

Judy Redfearn

Biotechnology

Looking ahead

Bethesda Research Laboratories (BRL), the Maryland company that shook the US biotechnology industry by announcing that cash-flow problems had made it necessary to make redundant more than a third of its 450 staff, seems — at least temporarily — to be out of the woods.

Company officials are confidently predicting that, despite the setback to its expansion plans, BRL will double this year the \$10 million sales which it achieved in 1981, thus maintaining the pattern of geometrical growth that the company has managed since its foundation in 1976. Comparable sales increases are being talked about for 1983 and 1984. Private investors in BRL have endorsed the line being pushed by Mr Stephen Turner, BRL's founder and president, that "leaner is fitter" — the argument, ironically, that is being used by President Reagan's science adviser, Dr Jay Keyworth, to justify cuts in federal support for research and development.

The company has just announced that it has been able to raise an extra \$5 million from its original investors, many of whom are based in Europe. According to Mr Turner, this should be enough to provide a stable base for a steady expansion of its product range, originally focused on restriction enzymes but subsequently expanded to include other biological

products such as monoclonal antibodies as well as diagnostic screening kits and a nucleic acid analyser.

Mr Turner blames the company's recent difficulties on the rapid drying up of venture capital in the United States in the past six months. Last year, anticipating that it would have little difficulty in raising an anticipated \$40 million through a public stock offering, the company laid the groundwork for an ambitious expansion programme, dividing its research products division into separate molecular biology and biological chemistry sections, and significantly increasing its research staff.

In recent months, however, a declining inflation rate and new tax incentives have taken some of the glamour out of venture capital dealings as an alternative to other, more traditional forms of investment. In addition, much of the available venture capital is said to have been soaked up by some of the early public offerings — such as that launched by Cetus a year ago, which managed to raise over \$100 million in the largest new issue ever experienced by Wall Street.

As a result, many of the small biotechnology companies launched in the past two or three years are experiencing severe difficulties in raising the capital they need to keep going, a situation that Mr Turner believes could last for at least another year. Several industry analysts expect that up to one half of the companies could disappear within the next few years, some being absorbed into larger

corporations, others filing for bankruptcy or actively seeking mergers. BRL claims to be in a stronger situation than some of the other companies since it is already selling products on the market.

"The days of over-expectation and pure speculation are behind us and the time has come to fine-tune operations and get down to the business of running a business," says Mr Turner. "That's what we have done at BRL."

Similar sentiments are expressed by Frederick R. Adler, a New York lawyer who has helped to set up a number of high technology companies in the computer field, and who has recently been appointed to BRL's board of directors. "The computer industry taught us that business can successfully serve science. We're now translating that lesson to biotechnology", he says.

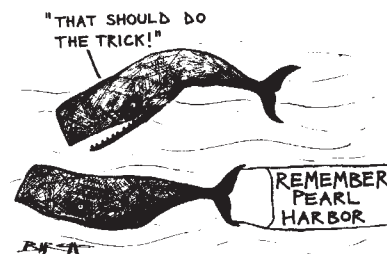
David Dickson

Sperm whale catch limits

No decision

A head-on clash between conservationist countries and Japan, the main whaling nation, was averted on Thursday when a special meeting in the United Kingdom of the International Whaling Commission (IWC) agreed to defer a decision on sperm whale catch limits until the annual general meeting in July. The conservationist countries abandoned their attempt to impose a total ban on sperm whaling after the scientific committee failed to produce a conclusive report.

Catch limits are usually set at the annual meeting of IWC. Last week's special meeting was convened because the scientific committee had not been able to offer unequivocal advice to the July 1981 meeting. Earlier this year, 32 scientists from 12 countries met in Cambridge to analyse sperm whale stocks. Computer tests were run using two rival models, one backed by the Japanese, the other developed by the International Institute of Environment and Development (IIED).



All except the Japanese scientists at last week's meeting supported the IIED model. However, the committee failed to offer any definite advice on catch limits. According to IWC criteria that stocks should be protected when they fall below ten per cent of maximum sustained yield (the maximum number of whales that can be harvested on an infinite basis without a decline in population), the IIED analysis indicated

Rorvik versus Bromhall

Washington

Hailed in 1978 on the book jacket as "the scientific breakthrough of the century", David Rorvik's book *In His Image: The cloning of a Human* was declared by a Philadelphia judge the same year to be "a hoax and a fraud". Rorvik returns to a Philadelphia court next week when Dr Derek Bromhall from the University of Oxford, who claims that his research was misused, will sue for damages against Rorvik and his publisher Lippincott and Co. (now owned by Harper and Row).

Mr Allan Friedman, Bromhall's lawyer and an associate of the firm Raynes, McCarty, Binder and Mundy, claims that Lippincott showed "reckless disregard for publishing ethics". He says that his client's case turns on two issues. The first is the claim that Rorvik invaded Dr Bromhall's privacy by quoting his cloning techniques in the text and by mentioning his name in the book's footnotes as a "personal communication" without approval. The second claim is that Mr Rorvik fraudulently acquired an abstract of Dr Bromhall's doctoral thesis by representing himself as a scientific researcher preparing a project on mammalian cloning.

Mr Rorvik's attorney, Samuel Klein of Kohn, Savett, Marian and Graf, claims that Bromhall's information did not

include "novel and unique" concepts, that the thesis was already on microfilm and that a summary had already appeared in the scientific literature (*Nature* 258, 719; 1975). Furthermore, he adds, there has never been disagreement over the fact that all references to Bromhall's work were fully accurate.

Mr Klein also plans to argue that in the single letter written by Mr Rorvik to Dr Bromhall, his client identified himself as a freelance journalist and that Dr Bromhall's presentation of his nine-page abstract was unsolicited. There was no oral communication between Rorvik and Bromhall at the time of this correspondence.

The trial promises to become heated, for neither party plans to settle out of court. Mr Klein also says he will question Dr Bromhall's wish to keep his distance from the book in light of the fact that Dr Bromhall "voluntarily associated himself" with the film *Boys of Brazil*, which portrayed the cloning of multiple Hitlers. But Friedman says that Bromhall's voluntary association with a film presented as fiction is very different from an involuntary association with a book whose author claimed it to be non-fiction.

The trial is expected to last several weeks and many scientists will make appearances for both sides.

Michael D. Stein

that males should be totally protected, but a catch of 831 females could be permitted. But the analysis also indicated that even with a zero catch the stocks would continue to decline for several years. The biological reason for this is that sperm whales are polygynous so that the past removal of breeding males, favoured by the whalers because of their large size, has resulted in a decline in the pregnancy rate of the stock.

Therefore, the Japanese argue that their current quota of 890 would make little difference to the sperm whale population, particularly since the number of exploitable whales was estimated by the IIED team to be around 200,000. But conservationist countries are keen to see a zero quota, pointing to the predicted decline in population with no fishing at all.

The inability of the special meeting to come up with catch limits was not so much the result of scientific equivocation as a political move to produce a compromise. The conservationist countries feared that had they enforced a quota unacceptable to the whaling nations, Japan would have exercised its right to object and both the procedure for cooperation and the whole credibility of IWC would have been seriously damaged.

The annual meeting of IWC in July may be faced with pressure for a ban from the conservationist countries, which will be in the majority. If Japan refuses to compromise and registers an objection, the conservationists would then have to try to apply the ultimate sanction — a US ban on Japanese fishing in its waters.

Jane Wynn

Environmental law

Count the risks

Washington

A subcommittee of the House of Representatives has taken a first step towards building scientific risk analysis into the federal government's regulations on environmental and safety hazards. A bill passed by a subcommittee of the Science and Technology Committee would establish a new programme under the White House's Office of Science and Technology Policy (OSTP) "to improve and facilitate" the use of risk analysis in regulatory decisions. The bill will now go to the full committee, whose chairman, Mr Don Fuqua of Florida, supports it.

The Reagan Administration is less welcoming, feeling that the goals of the proposed law could more simply be attained by rationalizing new and existing regulations. But according to OSTP, the Administration will probably find the bill "acceptable" as long as certain amendments are made.

Various members of the Administration argued in their testimony to the subcommittee that they have already taken steps to introduce a more "rational" approach to regulation. Last February, for example, President Reagan put out an executive order requiring all proposed new regulations to be accompanied by a cost-benefit analysis demonstrating that they represented the most cost-effective way of reaching their declared goals. The White House has also announced that the Inter-

agency Regulatory Liaison Group, established under President Carter to coordinate actions by different agencies, would be reconstituted as a new committee under the chairmanship of the President's Science Advisor, Dr Jay Keyworth, with the task of improving the scientific basis of regulatory decision-making.

Such changes have not satisfied the members of the House committee. Thus the new bill would assign to OSTP the specific role of establishing a mechanism for coordinating the risk analysis programmes among the participating federal agencies, primarily the Consumer Product Safety Commission, the Food and Drug Administration, the Environmental Protection Agency and the Department of Labor's Occupational Safety and Health Administration. And the National Science Foundation would be required to draw up a programme of research to make the use of risk analysis more effective. In addition, the various federal agencies would each be required to undertake a "prototypical risk analysis study".

Various powerful lobby groups, representing for example the agricultural or the nuclear power industry, have long been pushing for revisions to regulatory decision-making along these lines. The bill is therefore unlikely to lack supporters if it is sent by the full committee to the floor of the House of Representatives. But it is also likely to generate substantial opposition. Many Democrats, particularly those with close links with the labour unions, see attempts to "rationalize" regulation as a smokescreen to legitimize less stringent health and safety controls.

The Administration is likely to stand aside from any such debates. Although it claims to have made significant progress behind the scenes in eliminating unnecessary or redundant regulations, it is becoming wary of taking highly-publicized actions that might be interpreted as "anti-regulatory" given the apparent continued public support for strict environmental and health and safety controls — and the importance of the congressional elections later this year.

In a related effort to reduce the impact of federal regulations, the US Senate last week approved a proposed new bill which would give Congress the power to overturn regulations being proposed by any federal agency. The bill is being resisted by the Reagan Administration, which sees it as an attempt by Congress to place limits on the flexibility of the executive branch. It has also received strong opposition from environmental and labour groups, who argue that it would provide vast scope for individual companies and industries to lobby Congress to prevent certain regulations from coming into effect. Supporters of the new ruling, however, argue that regulatory agencies frequently exceed their congressional mandates, and that checks are now needed to limit their autonomy.

David Dickson

Join the queue for monoclonals

St Louis

The Monsanto Corporation of St Louis, Missouri, has made a multi-million dollar agreement with nearby Washington University to produce monoclonal antibodies, the second such agreement the university has made with the chemical industry in the past six months.

Monsanto says it has agreed to put \$1.8 million into research projects at the university during the next three years. No contract has so far been signed between the university and Monsanto but the deal is expected to be similar to that made with Mallinckrodt Inc., also of St Louis. Last September, Mallinckrodt committed \$3.9 million to monoclonal antibody research at Washington University, the largest industry agreement ever made with an American university for this type of research.

Mallinckrodt's three-year commitment is a major one for the company, which is economizing in other areas. But despite uncertainties about patents, monoclonal antibodies hold out enormous commercial promise. Dr Thomas O. Oesterling, Mallinckrodt's

vice-president for research and development, says his company's primary interest is in *in vitro* diagnosis, where monoclonal antibodies allow much improved standardization in diagnostic kits, but it is looking at other uses. "A monoclonal antibody could carry an isotope for imaging to a target cell for *in vivo* diagnosis", and from there it is "a logical extension to therapy".

The contract between Mallinckrodt and Washington University provides for the university to hold the patents for useful products of the joint venture, but for Mallinckrodt to have the right to license them. The university's share of royalties is dependent on the success of the product on the market.

One aspect that is covered by the arrangement with Mallinckrodt is the right of publication. Individual investigators do not profit personally from an invention, although some of the royalties will be ploughed back into research in the individual's laboratory, but the individual does retain the right to publication, with the proviso that the company can screen for patentable material.

Karen Freeman

Making waves

Following hard on the heels of Amersham International, another "public" company — the Hydraulic Research Station at Wallingford, Oxfordshire — is to go "private" on 1 April, with Dr John Weare as managing director and chief executive.

All of the present staff will be seconded from the civil service to the company for one year, after which 80 per cent (about 200 people) are likely to be offered long-term contracts.

The transfer was not without its hitches. Staff at first resisted the move fearing that "privatization" would mean loss of government backing and lead to a lessening of the station's world-wide reputation. In fact, no drastic cutbacks are planned and the general feeling is that, freed from government constraints, the company will be better able to respond swiftly and competently to the needs of the civil engineering industry.

Sara Nash

Indian science education

Falling behind

New Delhi

In a major new exercise, Indian government circles concerned with science education are trying to find resources equivalent to \$55 million for the next three years to strengthen the science education base of the universities.

The government realizes that lack of support has allowed the university science system to run down and that this trend, if continued, may prove irreversible. The role of universities as advanced centres of teaching and research has been eroded by the rapid expansion in the number of institutions and students without a corresponding increase in the necessary facilities.

The Science Advisory Committee to the Cabinet (SACC) has long felt that the resources of the universities should be augmented as this would not only benefit universities but also assist the major scientific agencies of the country and industrial enterprises. The scientific manpower requirements of these agencies will be met by the universities. SACC recommended that central and state governments and industrial companies should combine to contribute about \$55 million to strengthen the science base of the universities. A detailed scheme is expected to be submitted to the cabinet for approval.

The Indian government also feels that the current pressure on the universities in terms of the enormous intake of science students should be reduced, and that equal opportunities should be provided for gainful employment for those who do not pursue careers in science after passing the school level examination. This system will

then provide the smaller number of science students with proper facilities. And as lack of instrumentation is a serious handicap for research in universities, the government is now considering stepping up instrument production.

It is widely believed also that the links of university research and national scientific agencies with public sector enterprises need to be strengthened. The present barriers between the two are impeding the country's scientific and technological progress.

Sunil Saraf

●Speaking at the invitation of the Science Policy Foundation in London last week, Mrs Indira Gandhi reasserted her commitment to the strengthening of Indian science and technology. Without internal strength, she said, the governments of developing nations cannot withstand the tendency of some nations to use the transfer of technology as an instrument of foreign aid. "We have not got out of one empire to get into another" was her reply when later questioned as to her country's relationship with the transnational corporations.

Self-reliance is also necessary, Mrs Gandhi said, because the problems of India are increasingly different from those of the developed nations. India needs to curb population growth, to find ways to grow crops in soil of low moisture and to prevent cholera and tuberculosis, not cancer and heart disease.

At the same time it is of immense benefit, even a necessity, for India to continue its own atomic energy, oceanographic and space programmes. "Our space effort is important for education and communication and deeper knowledge of the monsoon, which rules our economic calendar."

While emphasizing the unacknowledged, reverse economic aid donated by emigre Indians to "Western" scientific progress, Mrs Gandhi said that opportunities for intellectually challenging work must be increased to keep talented Indian scientists in the services of their own people.

Peter Newmark

National Research Council

Changes ahead

Washington

The US National Academy of Sciences is carrying out a major reshuffle of the complex organizational structure of its National Research Council (NRC). The purpose, according to the academy's new president, Dr Frank Press, is "to meet the opportunities ahead of us, to promote greater efficiency in our ability to respond to governmental requests and to meet changing economic circumstances".

NRC, the operational arm of the academy, provides advice to and carries out research on a contract basis for the federal government and other

organizations. Since 1973, it has operated through four assemblies — each based on a single discipline or set of disciplines — and four multidisciplinary commissions.

This arrangement was introduced to improve NRC's ability to tackle policy questions that frequently cross disciplinary boundaries. In practice, however, there has frequently been confusion over the relative responsibilities of the various assemblies and commissions — a situation which Dr Press hopes the new structure will avoid.

Three of the assemblies will therefore be merged with three of the commissions. The Assembly of Behavioral and Social Sciences will be merged with the Commission on Human Resources to become the Commission on Behavioral, Social Sciences and Education; the Assembly of Engineering will merge with the Commission on Sociotechnical Systems to become the Commission on Engineering and Social Systems; and the Assembly on Mathematical and Physical Sciences will join with the Commission on Natural Resources to become the Commission on Physical Sciences and Resources.

The future of the fourth assembly, the Assembly of Life Sciences, some of whose work currently overlaps with that of the Institute of Medicine, will be discussed at the next meeting of NRC's governing board which takes place on 3 April. Any changes to the constitution of the Institute of Medicine would have to be approved by its members.

The fourth commission, on international relations, will be transformed into a new Office of International Affairs, whose executive director will be Dr Victor Rabinowitch. The activities of the office will be overseen by a panel comprising the foreign secretary of the academy, Dr Walter Rosenblith, the foreign secretary of the National Academy of Engineering, the president of the Institute of Medicine, and Dr Rabinowitch; this panel will also undertake policy formulation for the whole of the academy complex.

The reorganization is part of a broad attempt by Dr Press to streamline the academy's activities and enable it to play a more active part in Washington science policy debates. One example of this new approach is a panel which is being set up under the Committee on Science and Public Policy (COSPP) to examine the implications of the closer links being drawn between US universities and the Department of Defense.

The panel will examine a range of issues raised by this enhanced relationship, from the application of export controls to unclassified research to broader impacts on academic freedom. Funding for the study has already been promised by the Defense Department itself, the National Science Foundation and the American Association for the Advancement of Science; NAS is seeking additional funds from outside sources.

David Dickson

CORRESPONDENCE

The coal of today

SIR — The content of the leading article "A strategy for coal" (*Nature* 25 February, p.636) indicates a view based on out-dated knowledge of the coal industry. The economic arguments used ignore, for example, the fact that British coal is the most competitive in the European Community. The latest published coal production cost comparisons (for 1980) are:

	£ per tonne
Belgium	61
France	45
West Germany	44
UK (1980/81)	35

The article mentions the design of microcircuits as if this was a world apart from coal mining. In fact, Britain's technologically advanced coal industry extensively uses computers, microprocessors, transducers, lasers and other modern aids. There is obviously no future in the microtechnology unless there are industries like coal to use the products. The National Coal Board and private enterprise manufacturers have successfully designed and introduced computerized control and monitoring systems for colliery operations and these are creating considerable interest overseas.

Longwall mining technology was pioneered in Britain and equipment sales remain significant export earners — and have resulted in record-breaking colliery performances in the United States, South Africa and Australia. There are many other examples showing that our indigenous coal production is serving Britain well — and it will need to do so in the future. Incidentally, the Australian exports mentioned in the *Nature* article include a high proportion of opencast coal which is also profitably produced in Britain. ROBERT HUNT
Dowty Group, Cheltenham, UK

Doves in false garb

SIR — Having read more than once your leading article of 18 February (p.542) under the condemnatory title above, I am still puzzled that you provide no evidence, by way of argument, to justify the statement "The claim by the anti-nuclear movement of professional support is mostly a sham". The occasion you were commenting upon, the Conference of Professions for World Disarmament and Development, convened under the auspices of the World Disarmament Campaign, made no claim to speak officially for the major professional societies. But the attendance of several hundred professional people, and the rapid growth of specialist bodies such as Scientists Against Nuclear Arms (SANA), Medical Campaign Against Nuclear Weapons, and Teachers for Peace suggests that there are many who do agree in general terms that "the professions as such have a responsibility to alert the general public to the great issues that confront society". You seek to deny this, yet you accept in the very next sentence that "there is a sense in which professional people, acting individually, may be held to shoulder an extra responsibility for giving wider currency to their conclusions about important issues on which they have some special knowledge".

Why the emphasis on acting *individually*?

Why do you consider it "not respectable" for scientists of different disciplines to come together through SANA to pool their varied expertise relevant to the effects of nuclear war on Britain, and then to make known their conclusions based on this special knowledge? Why try to belittle the work of the Medical Campaign Against Nuclear Weapons and the Medical Association for the Prevention of War, who point out to their colleagues that nuclear war would be a medical catastrophe beyond reach of any cure, so that it becomes a *professional* duty to seek its prevention?

You complain that the major professional bodies "are needlessly indifferent to important public issues well within the spheres in which their professional competence could command respect". Yet you condemn, by implication, the work of the British Medical Association to study the medical effects of nuclear war, and the efforts within the teaching profession to direct attention to the role that education plays in forming public attitudes to other countries and to conflicts within and between countries. Do you not accept, for your own profession of journalism, some responsibility for the way in which the press and television treat major issues such as nuclear war and thus influence the formation of public opinions?

CHRISTOPHER MEREDITH
(Secretary, Scientists Against
Milton Keynes, UK Nuclear Arms)

SIR — The leading article on the meeting of professional groups concerned with supporting nuclear disarmament makes a number of astonishing assertions (*Nature* 18 February, p.542). For instance it seems to question the right of the medical profession to express an opinion on the issue, claiming that "Physicians have been consistently indifferent to the quality of health care (in Britain)". This suggestion is as insulting to the medical profession as it is unfounded.

The health care professions should and do have a special knowledge of the medical effects of nuclear weapons as have other professional bodies, such as architects, specialized knowledge of such matters as affect their sphere. It would seem entirely understandable, and we would submit entirely proper, that such organizations should undertake to publicize as widely as possible the full effects of the use of nuclear weapons.

The rapidly expanding Medical Campaign Against Nuclear Weapons, and no doubt the other professional groups represented at the meeting discussed in the article, reflect the widespread concern over the proliferation of nuclear weapons and the apparent increasing risk of their use. In the United States, professional bodies voice similar concerns. Thus a recent report of the American Medical Association stated "There is no adequate medical response to a nuclear holocaust".

Doctors and other professional organizations not only have a right but a duty to speak out on the subject of nuclear weapons and nuclear warfare. They do so in order to bring the facts to the attention of their colleagues, politicians and the general public and not, in the remarkable words of the leader, to capture any "mediaeval mystique".

M. HARTOG, J.H. BAUMER,
P.J. FLEMING, M.J. HALL
University of Bristol, UK

SIR — The views expressed in your leading article entitled "Doves in false garb" (*Nature* 18 February, p.542) were put forward provocatively enough to require reply.

You have allowed that "there is a sense in which professional people, acting individually, may be held to shoulder an extra responsibility for giving wider currency to their conclusions about important issues on which they have some special knowledge". Why must they be "acting individually"? Why, that is, when medical people act together, do you decry this as their "attempt to invest their legitimate causes with spurious authority . . . a deceit"?

Medical people certainly have no entitlement to speak with any lofty authority. But we have seen people die of burns and crush injuries, and have had to repair terrible lacerations. There is always at such times the question "why need this have happened?", and physicians, surgeons and general practitioners alike bear, or should bear, prevention constantly in mind. For epidemiology prevention is the *raison d'être*. It means searching for any predisposing causes of disease or injury and proclaiming them when they are found. For many of us it is no longer tenable to act in this way where, say, chemical carcinogenesis, dietary causes of disease or accidents in the home are concerned, but to remain mute on the dangers of nuclear detonations. Psychiatrists especially are left with few illusions about human fallibility.

STEWART BRITTEN
London, UK

A final word

SIR — "The creationists may have lost the battle in Arkansas but they are unlikely to abandon the war on which they are engaged" (*Nature*, 14 January, p.85).

Your journal has, for several months, lent itself as a battleground for this verbal brawl by continuing to publish letters from both sides, many of which do not (and cannot) contain any elements of scientific reasoning. This correspondence has degenerated into a discussion of the Bible as a source of artistic inspiration (A.J. Hollin, *Nature*, 18 February, p.548) and such fine points as the positioning of a comma in Luke's gospel (F.W. Cousins, *Nature*, 11 February, p.452). These authors clearly have a sense of the absurd.

Nature is a journal with a large and mixed audience which serves not only to publish important scientific findings, but also to inform scientists about political issues which are of particular interest to them. The Arkansas trial was clearly one such issue. That trial is now over, and scientists wishing to continue the discussion should now look to do so from a platform where they might be better able to inform and enlighten the general public. Does Dr Salthe (*Nature*, 11 February, p.452) really need to point out to a scientific readership the folly of the statement that "The scientific community does not consider origins of life a part of evolutionary theory"?

Serious discussion of mechanisms involved in evolution has a place in this journal. Advocates of God and *Genesis* should air their views elsewhere.

JANE CALVERT
University of Alabama, USA

This correspondence is now closed — EDITOR

An end to the search for new drugs?

M. Weatherall*

THE discovery of new drugs is taking longer and becoming less productive¹⁻³. In the United Kingdom the average time between first publication (itself usually years rather than months after studies begin) and marketing increased from 6 years in 1965 to 10 years in 1978⁴. In the United States the average time between filing an "Investigational New Drug" (IND) — in effect advising the authorities of an impending trial in man — and approval by the authorities of a drug for general use increased from about 3½ years in 1967 to 9 years in 1976⁵. One British pharmaceutical company has found⁶ that between 1972 and 1978, the number of animals used in safety testing increased from 600 to 1,200 or more and that the number of man-days spent by trained staff on a single non-rodent study increased from 275 to 480 despite improved working methods which saved 85 man-days. Unpublished figures from the Wellcome Foundation show that the cost of studies necessary before clinical trial of a drug intended for long-term use in man rose between 1965 and 1979 by a factor of 3.2 for toxicology and 5.2 for pharmacokinetics, even after correction for inflation.

The money for this work has to be found somewhere. The more that is spent on bringing one drug into general use, the fewer the total number of drugs that can be studied and the more necessary it becomes to concentrate on common diseases where the prospects of a financial return are greatest. Drugs for rare diseases — sometimes called "orphan" drugs — such as triethylene tetramine for Wilson's disease⁷ or L-5-hydroxytryptophan for myoclonus⁸, are not commercially viable and so must be prepared in a physician's laboratory and encapsulated in a hospital pharmacy⁷ if patients are not to be allowed to die.

It seems probable that financial pressures have already reduced the total research in the United Kingdom directed at finding new drugs. Cavalla⁹ lists nine companies which have closed their research and development groups, with redundancies or reorganization.

It can, of course, be argued that drugs are useless if not positively dangerous, and that the resources used in drug research would be much better spent on measures of more general benefit to the health of the community. There is a need to balance drug development against spending on

community health, preventive medicine and relevant education. But as the funds come from different sources, striking a balance is necessarily difficult. Making drug development a government responsibility is not, however, an answer. A government concerned to live within its income from taxation would face the same problems as industry, and would perhaps be even more reluctant to undertake the risks of financial loss associated with speculative research, the problems of making orphan drugs available for uncommon but killing diseases and so on. Ultimately, health can be achieved and maintained only by work. The limit of health care is the amount of work which is put into it, and unless the work is unpaid, the limit is economic.

Meanwhile, until the balancing act is achieved and the public desire for medication¹⁰ abates, drugs will continue to be a major element in therapeutics and better drugs will be sought. No drug is without its hazards. The one prediction that can con-

evidence about the effects of drugs used by specialists in closely supervised patients. The effects may differ greatly when a drug is released for widespread and unregulated use. Information about the overall prescribing of drugs is of little help in assessing benefit, while the proportion of prescribed drugs actually consumed by patients is usually not known. The reporting of adverse reactions is patchy and biased, and the fashion for expressing alarm about iatrogenic disease needs to be tempered by a careful appraisal of how the sufferers would have fared without the treatment.

In particular instances, thorough studies allow an assessment of risk. Thus immunosuppressive treatment, with azathioprine, chlorambucil or cyclophosphamide, is used to prevent rejection of transplanted kidneys. These treatments carry a 1.3% risk of tumour induction and 0.4% risk of mortality within the period of observation¹³. But this risk has been estimated only after ten years of study. It could not

Public demand for totally safe drugs has led to excessive, costly and misleading toxicity testing. Such testing drains the resources which have been available for discovering much needed new drugs. Better public understanding of the limitations of toxicity testing and the hazards of medication is sorely needed in order to improve the prospects for diseases still needing effective treatments.

fidently be made about any new drug is that it will have unwanted effects, and dangers if misused¹¹. Moreover, it is usually difficult to assess what contribution drugs have made to the reduction of ill health and prolongation of life, when sanitation and nutrition have improved at the same time. Yet in a variety of circumstances, drugs are unequivocally saving individual lives¹², as with the use of antibiotics in a wide range of acute infectious diseases, and of vaccines in the elimination of smallpox throughout the world and in the minimization of diphtheria and poliomyelitis in many countries. Replacement therapy prolongs life unequivocally: no one could seriously maintain that all diabetics could remain healthy without insulin. There is no doubt that *some* drugs are vitally necessary.

There is also no doubt that over a wide range of drugs, the evaluation of therapeutic benefit is extremely difficult. Difficulties arise when prolongation of life and easement of disability are the main expectations. Mortality statistics in more developed countries are good, but morbidity statistics are variable and, except for notifiable diseases, hard to come by. Clinical trials seldom extend over more than a year or so, and by their nature give

have been estimated when renal transplantation began. Had it been possible to know it, and had it been added to the operative risks and other hazards, would it have been a reason for not embarking on the procedure and would most of the people now living with transplanted kidneys have been allowed to succumb to their disease?

Drugs are tools which function by modifying the processes of life, and they cannot be labelled good or bad without considerable knowledge of the circumstances in which they are actually used. For many diseases, the drugs available now are clearly inadequate to restore health or even minimize discomfort and disability. Allergy, arthritis, cancer, cardiovascular disease, congenital abnormalities, psychiatric, neurological and a variety of tropical diseases all await effective treatments. There is a rapid growth in knowledge about the biochemical disorders and genetic background of many of these conditions, and either from such knowledge or by more empirical methods, new drugs continue to be found. But there is a drug lag in countries which have the most sophisticated regulatory arrangements¹⁴, and a virtual halt to the discovery of new drugs for poorer and less healthy nations¹⁵.

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One reason for the decline in innovation may be the difficulty of devising suitable laboratory experiments for identifying drugs for the unconquered diseases. When a disease is due to a known infecting organism, compounds can be selected according to knowledge of the biochemistry of the organism, or empirically. Such compounds can be tested against the organism *in vitro* and *in vivo*, and the most effective selected for further study. The search may be long, but the route is clear. It has led to the whole range of chemotherapeutic drugs and antibiotics which have outstanding powers of saving human and animal lives. But this field is largely worked out, except for the tropical diseases. It will grow again in importance, as more drug-resistant organisms evolve. The resources for such discoveries are still an important part of the infrastructure of public health.

Drug searches

For diseases not known to be due to an infecting organism, some other clue is necessary to start the search for useful new drugs. When a particular substance is shown to be lacking, its replacement is indicated, as with L-dopa for Parkinson's disease¹⁶ and, earlier, in the various endocrine gland failures. New local hormones or mediators continue to be discovered, but it remains to be seen how extensive the practical therapeutic consequences will be of the great body of recent work on prostaglandins, leukotrienes and neuropeptides. There are advances to be made in neuropharmacology as the relationships between disorders of synaptic transmission and mental disease become understood. At present the field is confused and confusing, and hampered by the lack of reliable animal models for essentially human disorders. So, too, diseases involving some ill-defined immunological disorder — rheumatoid arthritis^{17,18}, allergies of all kinds, perhaps multiple sclerosis¹⁹ — must either await biochemical understanding or rely on animal models justified in the main because they give positive results with agents already known to be effective in the human disease. This is a route to me-too drugs (not necessarily worthless) and to much waste of resources on false positives, but it is not likely to lead to great innovations in therapy.

The other major strategy for discovering new drugs is to pursue the detail of a physiological or biochemical system and to exploit its practical application. This is the natural course of academic scientists, and has led to such important agents as insulin and dimercaprol. In the past thirty years, however, the practical application by academic scientists of their discoveries has declined²⁰, partly because the resources now needed for development are completely beyond the range of university facilities and partly because increasing specialization within universities has reduced the cross-links between approp-

riate specialists, especially organic chemists with pharmacologists, and pharmacologists with clinicians²¹. The fundamental approach has been pursued more successfully in industry, notably in Hitchings' work²² on purine and pyrimidine metabolism and inhibitory analogues, which led to drugs as diverse as the antimalarial pyrimethamine, the anti-neoplastic agent 6-mercaptopurine, the immunosuppressant azathioprine, allopurinol for gout and the antibacterial trimethoprim. More broadly, all the developments of drugs associated with neuro-effector and synaptic transmission rest on the fundamental work initiated by Dale at Wellcome Research Laboratories, extended in countless other institutes and applied to practical ends by many pharmaceutical manufacturers.

A major development of this kind is necessarily a long haul and involves extensive investment of resources in a concept, the therapeutic merit of which can be judged only at the end of the road. This kind of investment requires circumstances of prosperity and reasonable security, and is hindered if the always limited resources for research are frittered away in unimaginative and invalid routines.

In a reasonable world, the increasing difficulties of discovery and the seriousness of the incurable diseases may be seen as good grounds for a more tolerant attitude to the hazards of therapy. Desperate ills need desperate remedies, but the wish for completely safe remedies has had and is having the gravest effects on drug discovery²³. The problem of finding valid laboratory models for the unsuspected toxic effects of drugs is just as difficult as the problem of devising methods for detecting therapeutic potential.

The major tragedies which have created public alarm and fear and which have led to the condemnation of drugs have arisen in special circumstances (pregnancy and thalidomide; consumption of amine-rich foods and monoamine oxidase inhibitors; Japanese life and clioquinol²⁴) or have been due to unexplained sensitivity in a minority of patients (triparanol²⁵, practolol^{26,27}, clozapine^{28,29}). Practolol, a β -blocking agent introduced for the control of cardiac arrhythmias and subsequently found valuable also in hypertension, is particularly notable for the thoroughness with which its toxicology was studied in animals, to the satisfaction of registration authorities. Nevertheless, adverse reactions, some serious, developed in a small proportion of patients who received the drug; the ill-effects included skin rashes, impairment of secretions and, most gravely, corneal damage sometimes leading to impairment or loss of vision (the "oculomucocutaneous syndrome").

None of the recognized laboratory procedures gave warning of the serious clinical syndrome nor, even with hindsight, has a procedure been discovered which would have predictive value. Other "idio-

syncrasies" are associated with particular genetic factors, as with reactions to suxamethonium³⁰, primaquine³¹, penicillamine³², sodium aurothiomalate¹⁸ and hydralazine³². When much more is known about individual biochemical variation and its genetic control, it may be possible to predict that particular drugs will be hazardous to particular individuals. But the animal models of orthodox toxicity testing give no basis for such subtle predictions.

The unsatisfactoriness of predicting adverse effects in humans from animal experiments has been well known for a long time³³⁻³⁶. Positive findings in animal experiments are also not always reliable evidence of harm to man. Usually, positive findings in experiments in animals are grounds for not giving a compound to human beings at all, but sometimes some toxic effect in animals is discovered after a drug is well established in clinical use. Furosemide, a well tolerated and valuable diuretic in man, causes severe liver necrosis in mice³⁷ because of a metabolite which is not formed to a serious extent in humans³⁸. The nucleoside 6-azauridine is tolerated for relatively long periods for cancer chemotherapy in man: in dogs, smaller doses than are acceptable in man produce potentially lethal bone marrow depression in 7-10 days³⁹. Intramuscular injection of iron sorbitol causes sarcomas at the site of injection in rats and rabbits⁴⁰. The implication for human therapeutics appeared serious⁴¹; but the nonspecificity of the effect in laboratory rodents was recognized⁴² and, nearly 20 years after the original observation in rats, only eight published cases could be found of tumours after intramuscular iron injection; their variety did not suggest a common origin from the injected material⁴³.

Species specificity

Every species has its own metabolic pattern, and no two species are likely to metabolize a drug identically⁴⁴. Small differences in the rate of conversion of drug to inactive, or to toxic, metabolite can have large effects on the concentration of active substance at the point of action. Most experiments to seek toxic effects in whole animals involve oral administration; differences in diet, gut physiology, rate of passage and liver enzymes raise serious questions about the relevance of findings in rats or mice to man. Compounds which are not absorbed in laboratory animals are not, with minor exceptions, ever tested in man. Nobody knows how many drugs, which would be useful in man, have been lost in this way. Similarly compounds toxic in laboratory animals at doses near the predicted therapeutic level do not receive trial in man, so it is never revealed whether they would actually have been harmful in man. Thus we lack the evidence of the false positive element in animal toxicology studies³⁹, so it is easy to give more weight to such studies than is justifiable.

The procedures⁴⁵ which became

established after thalidomide whereby large numbers of animals were fed large amounts of drug for a long time, have become ritual "routine tests of limited value and governed by regulations rather than by rational thought"⁴⁶. Reports on the results are submitted to regulatory bodies in confidence and seldom published. The experiments are rarely repeated by independent workers: nobody is motivated to find the very considerable resources needed to do so.

Supplementary tests — for effects in pregnancy and on fertility, for carcinogenicity, for mutagenicity — grow in number but not in validity, and the search for quicker and cheaper tests, notably using mutagenicity in bacteria *in vitro* as evidence of carcinogenicity *in vivo*, has added to the obstacles, uncertainties and expense of developing drugs. As with the primary toxicity tests, positive findings are a bar to further progress, and to validating the test, so that the procedures acquire a possibly unmerited aura of respectability. The greater the number of tests, the greater the chance of a false positive and the loss of a useful drug.

There are good reasons for special caution in applying results of experiments *in vitro* to conditions of practical use, especially when massive concentrations are used. In mutagenicity tests, for example, considerable adjustment of experimental conditions appears to be necessary to obtain a positive result with a chosen compound. Ashby and Styles⁴⁷ list 14 factors which can be varied in, and influence the outcome of, the Ames test, which is widely accepted as a standard basic procedure. By variation of any one or more of these factors, the sensitivity of the test can be altered, sometimes by as much as 400-fold. For the innocent who wish only for a black or white answer, the outcome can be readily changed from positive to negative or vice versa.

***In vitro* reliability**

In other words, there is a considerable scope for adjusting the test to give the required answer with any specified compound, and it is a great help in selecting test conditions to know what answer is wanted. Ames and Hooper⁴⁸ observe that "using rat liver homogenate as a model for a rat's metabolism of foreign chemicals is a reasonable first approximation". But, *in vivo*, compounds are metabolized and excreted, either unchanged or as metabolites. It is as important to mimic the mechanisms of inactivation as of activation if misleading results are to be avoided. The actual concentrations to which DNA is exposed in real life are quite unpredictable from concentrations produced in such experiments *in vitro*. The use of human instead of rat liver would add marginally to the predictive value of such bacterial tests for human mutagenesis. The addition of further enzyme systems might be better still, but would not overcome the problem

of relating the tests to real life concentrations and duration of exposure.

Indeed, unless mutagenicity *in vivo* differs from all known pharmacological effects, it will be affected by the concentration of the agent to which the DNA is exposed and by the length of time and by the frequency of exposure, none of which can be properly assessed from data in bacteria *in vitro*. Even if mammalian DNA is damaged in the same way as the DNA of bacteria in a test situation, repair mechanisms are well known in mammals⁴⁹, and may annul any damage so caused. The extent of their protective role has yet to be established. But no compound has been proved to be a mutagen in man and, 35 years after the massive radiation of the people of Hiroshima and Nagasaki, no deleterious heritable mutations have been recognized, so the protection afforded by repair mechanisms has had a substantial trial and appears to be pretty good.

Finally "the" correlation between mutagenicity (in bacterial tests) and carcinogenicity in humans is anything but simple. Many more carcinogens are known in rats than in humans and demonstration of carcinogenesis in rats sometimes depends on notably large and repeated doses of the substance under test⁵⁰. The importance of a carcinogen, as of any other toxic or therapeutic agent, depends on the concentration and persistence of the substance at the site of action, or more roughly on the dose and duration of exposure. Disregard of quantitative considerations is the first step — and it can be a very long step indeed — towards nonsensical prediction and the abandonment of useful substances on ridiculous grounds⁵⁰.

The problem remains of deciding what weight to attach to results of ominous but uncertain significance. The quicker and cheaper a test, the greater the number of substances on which suspicion may be cast. The list already includes the essential antimalarial drug chloroquine⁵¹, and such more familiar substances as caffeine⁵², tap water⁵³ and oxygen⁵⁴. Common sense prevents alarm about oxygen, which does, after all, damage the lungs and central nervous system of adults⁵⁵ and cause blindness in premature infants⁵⁶ at only five times the normally accepted concentration. Identification of a hypothetical carcinogen or co-carcinogen among the solids dissolved in some samples of tap water is less certain to lead to a major advance in public health than to promote expensive but nugatory research, paid for by charity or the public purse.

Coffee drinkers are no more likely than smokers to be diverted from their habits. The epidemiological evidence of association between coffee drinking and carcinoma of the pancreas⁵⁷ requires further investigation to establish whether one causes the other or whether both arise from an unidentified cause, but such an investigation will at least relate directly to a human problem. Will regulatory

authorities become concerned with chloroquine regardless of its place in the control of malaria? The regulatory authorities mostly operate in the rich countries, which can afford them and which are relatively untroubled by malaria. If chloroquine were banned as a mutagen in any western country, would its further supply to less developed countries appear to be morally responsible? The difficulties of preventing "character assassination" of a drug are great, witness the problems of phenacetin⁵⁸, metronidazole⁵⁹⁻⁶¹ and Debendox⁶², but the resulting losses to human health may last for ever.

What really matters is what happens in everyday life. Recognition of the fallibility of laboratory studies has led to a welcome demand for surveillance of the effects of drugs in ordinary use²³. However, it is very difficult to measure the incidence of adverse drug reactions^{63,64}, most of which are infrequent and occur in patients who are usually the recipients of several different drugs — and who may not necessarily consume the drugs prescribed for them. Naturally, a drug suspected of causing a reaction is withdrawn and many reactions subside. To give the suspected drug again so as to discover whether its toxic effect is reproducible, is hazardous and unethical, so evidence of individual cases is of very poor scientific quality.

Simplify regulation

Various schemes of surveillance have been proposed^{65,66}, by which it is hoped that quantity of evidence will overcome the weaknesses of separate observations. The accumulation of much data is essential, and the costs are substantial. If the price of drugs is not to rise, savings must be made elsewhere⁶⁴. One obvious area is in preclinical evaluation and its administrative costs. The recent simplification in requirements in the United Kingdom for a clinical trial certificate⁶⁷ are therefore welcome, though the financial savings will be modest. Studies of post-marketing surveillance are desirable, but if expensive schemes add to the economic barriers of discovering new drugs, they may yet achieve more social loss than benefit.

A basic difficulty lies in public opinion, and in the public belief that drugs can be, and ought to be, completely safe. Public conception both of the probability and of the severity of a risk is complex and not well understood. A single large-scale tragedy evokes much fear, as after an air crash or the sudden revelation that many deformed babies had been born after the use of thalidomide, whereas a continuous series of small tragedies, as in road accidents, is treated apathetically^{68,69}. Most people understand that a surgical operation carries a finite risk, and face with more or less equanimity the remote possibility of death on the operating table or some other ill-fortune. But few appreciate a similar remote risk from consuming a drug, and the majority are correspondingly shocked

when such an event occurs. The public view might be expressed in such words as "everything that could have been done to make a drug safe should have been done; even if tests are not very reliable, they had better be negative". A better understanding of the factors which create such views is needed if real safety and perceived safety can both be attained. Comparisons of the risk of taking the drug with the risk of omitting it from treatment are hard to make and harder still to present convincingly. And, ironically, it is not only wicked to do harm with a drug but often unethical to omit to administer it.

In this situation, the role of regulatory authorities is as unenviable as the position of the manufacturers. The regulatory authorities exist to protect the public. The losses incurred by delaying or banning new agents are neither obvious nor, as cold statistics, heart-rending. Occasionally, however, they have been measured. Delay in the introduction of the anticonvulsant drug sodium valproate on to the market in the United States has been estimated to have subjected American patients to approximately one million unnecessary seizures a year at a cost of approximately \$200 million a year⁷⁰. Delay in the introduction of suitable anti-arrhythmic agents has been estimated to cost 10,000 deaths a year in the United States⁷¹.

Other substantial delays in introducing new drugs into the United States as compared with the United Kingdom have been carefully documented: 7 years for the first β -blocking agent for hypertension, 5 and 3½ years respectively for cromoglycate and inhaled beclomethasone for asthma, 5 years for cotrimoxazole for urinary infections⁷⁰. It is difficult to estimate in every instance how many episodes of illness would have been prevented or treated more effectively if the drugs had been available. But presumably a sufficient quantity of evidence has accumulated in each case to justify the widespread use of the drugs. Why else did the Food and Drug Administration (FDA), in the end approve their use?

Public pressures

Unhappily, the balance sheet is not calculated solely on the best estimates of benefit and risk. Public opinion and political pressure weigh heavily against any suspicion of harm and are comparatively indifferent to benefits unless they are near miraculous. Triazurine (azaribine) has been described by the director of the FDA's Bureau of Drugs as "a classic example of a highly effective drug which is useful to a small number of patients with a serious disease (psoriasis) but which also carries a serious risk of harm (thrombo-embolism) in a fraction of those who take it" (J. R. Crout cited in ref. 72). It was released by FDA and later withdrawn, and its licensing became the subject of a congressional enquiry. Shubin's account⁷² of the proceedings raises serious questions about the

exercise of medical judgement in a democratic society: clearly safety as a motive over-ruled any question of medical benefit.

Science does not flourish in the political arena, and the problem becomes philosophical: is it better to take risks in the hope of benefit, or to prohibit expected benefits because of an uncertain risk? Specific regulations are not as a rule unreasonable; it is the cumulation of requirements, their inconsistency between nations and their promotion of the mechanical check list as a substitute for reason and responsibility which achieves undoubted harm and uncertain good.

No manufacturer wishes to put unsafe drugs on the market, and it is natural to do what tests appear feasible to ensure safety, but the multiplication of tests of uncertain validity in no way makes them cumulatively any more reliable. It only increases the probability of rejecting useful substances on bogus grounds. It adds to the cost of developing new drugs, which is another way of saying that it diverts resources from discovery to supposed safety testing or, worse, to extensive experiments required to refute some suspicion raised on fashionable grounds but of dubious validity. And it delays the production of new remedies, during which time patients continue to suffer and die for lack of adequate therapy. One must have

sympathy for the industry and regulatory authorities in attempting the impossible tasks of giving complete protection against drug injuries, satisfying the public and defusing those who would make political or financial benefit by exploiting such hazards. But there is no excuse for any parties promoting scientifically worthless or dubious procedures.

Resources are limited, and the diversion of resources in pursuit of safety inevitably reduces the resources available for more constructive purposes. *Primum non nocere* is a good doctrine for medical students to assimilate, but it is going beyond common sense to refrain from using a substance which may save a hundred lives for every one person whom it harms. It is more than ten years since the words⁷³ were published "... the public and its administrative watchdogs have the choice between some risks or no new drugs at all. No problems are avoided by the multiplication of unvalidated tests". But the habit persists of over-reacting to alarming information, of seeking safety without counting the cost of approaching it, of failing to preserve a balanced and scientifically acceptable appraisal of evidence of risk and of benefit. How long will it take for the experts to gain courage and for the unattractive message to be presented to and understood by the public at large? □

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NEWS AND VIEWS

A new class of contraceptives?

from H.M. Fraser

In the last three years there has been considerable progress towards new contraception based on the use of analogues of luteinizing hormone-releasing hormone (LHRH). They should have an important place as contraceptive agents, for they are innocent of the side effects of steroidal methods, have no metabolic effects and are rapidly inactivated¹.

The characterization in 1971 of the 10 amino acid sequence of LHRH, by Schally and Guillemin, has led to the synthesis of progressively more potent antagonists and agonists (see the figure). These can inhibit reproductive functions by decreasing or increasing the LHRH stimulus to the gonadotrophic cells in the anterior pituitary gland, modulating the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Surprisingly, the agonists (originally developed to treat infertility) have the more dramatic inhibitory effects. Their effects were first noticed during toxicity trials in rats with high doses of LHRH and its agonists; given daily, they disrupted oestrous cycles, inhibited luteal function and pregnancy and, in males, caused involution of the seminal vesicles and prostate glands and impaired spermatogenesis¹⁻³.

The inhibitory effects of low-dose agonist treatment have since been extended to monkeys^{4,5} and women⁶⁻⁹. Early attempts to stimulate ovulation in anovular women with chronic therapy led to the finding that pituitary responsiveness is decreased, not increased. This suggested that LHRH might be used to prevent ovulation in normal women, and early studies suggested at least three possible procedures. Ovulation could be inhibited by injecting a LHRH agonist daily throughout the cycle (5 µg per day)⁶, an inadequate luteal phase could be induced when agonist was given at mid cycle (100 µg per day)⁷ and luteolysis could be provoked by giving agonist for 2 days in the late luteal phase (50 µg per day)^{8,9}.

The search for luteolytic agents has been one of the major goals of the contraceptive developers. One obvious advantage of such agents is that a woman would have an otherwise normal cycle. The fact that a luteolysin need only be administered at the end of a cycle would reduce the risk of

toxicity and side effects and also lower cost. But luteolytic agents are often ineffectual when conception has occurred, for their effects can then be offset by the stimulatory effects of human chorionic gonadotrophin (hCG) produced by the trophoblast¹⁰.

There is, however, no question that fertility is prevented when the agonist is administered daily to prevent ovulation. In clinical trials on 30 women in Uppsala¹¹⁻¹³ and 60 women in Berlin^{14,15} using agonist as a contraceptive for 3-12 months, no pregnancies occurred and there were no problems with reversibility. Daily injections are obviously impracticable but agonist can be administered in a nasal spray, although larger quantities (400-600 µg) must then be given.

Continual administration of LHRH agonist produces considerable changes in ovarian steroid production. Because ovulation is inhibited, the progesterone normally produced by the corpus luteum is absent, but this by itself is of little consequence. Variable and unpredictable changes in the most potent female sex steroid, oestradiol-17β, are much more important. It is essential that the oestrogen does not vary outside certain limits: too little, and women may develop menopausal symptoms, too much, in the absence of progesterone, and there will be proliferation of the endometrial lining of the uterus, predisposing it to hyperplastic change.

In about one-quarter of the women studied, the agonist prevented follicular development as well as ovulation, as indicated by consistently low concentrations of oestradiol in the blood¹¹⁻¹⁵. Thus there is little stimulation of the endometrium and women are virtually amenorrhoeic. Fortunately oestrogen was still present in sufficient amounts to avoid problems of oestrogen deficiency in all but a few women.

In the remaining women, ovarian function was less than fully suppressed, follicles still developed and oestrogen rose at varying intervals of time to stimulate endometrial growth. When the oestrogen

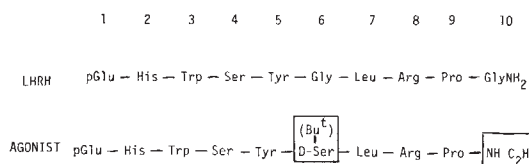
levels fall in the absence of ovulation, endometrial bleeding occurs. In a few women, who are presumably even less suppressed, these rises in oestrogen are followed by a small rise in progesterone indicating either ovulation with an inadequate luteal phase or luteinization of an unruptured follicle^{14,15}. In these women, bleeding is more regular.

Many women would object to such alterations of their monthly menstrual patterns. Those who become amenorrhoeic may welcome the opportunity to be free of periods once contraceptive efficiency is ensured.

The safety of chronic agonist treatment hinges on its effects on the endometrium. In the Swedish¹² and Berlin study^{14,15}, and in macaque monkeys treated with agonist for more than a year¹⁶, there was a spectrum of endometrial activity ranging from inactivity (usually when blood oestrogen was low) to marked proliferation. Although a chronically proliferative endometrium is undesirable, it will be of interest to compare endometrial activity with that of lactational amenorrhoea (low oestrogen, no progesterone) in which many women used to spend much of their reproductive lives without adverse effects.

Regular menstrual bleeding may be achieved by using lower doses of agonist¹⁵ or by confining treatment to the early follicular phase²¹, to permit ovulation followed by a defective corpus luteum. This should be incompatible with implantation but there may be some risk of an occasional fertile cycle. The best approach might be to provide exogenous progesterone for a few days at the end of a period of agonist treatment, priming the endometrium and inducing menstruation when withdrawn. Preliminary results of such a regimen are encouraging¹⁷ and although supplementation with progesterone detracts from the idea of completely avoiding exogenous steroids, it is not a return to the 'pill' since no oestrogen is given and progesterone would be administered for a short time only.

The amino acid sequence of LHRH and a widely used agonist.



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The use of LHRH agonist as a contraceptive is probably most attractive for women over 35 who do not wish to take the 'pill' because of its minor side effects, as well as the increased risk of cardiovascular problems. The agonist might also prove ideal for lactating women, who would more willingly accept the prolongation of their lactational amenorrhoea, and in whom the combined steroidal oral contraceptive is contraindicated because of its adverse effects on lactation. Agonists used as contraceptives may also, by lowering oestrogen and inducing amenorrhoea, benefit women with menorrhagia or endometriosis while the inhibition of the cyclic progesterone rise may help relieve some symptoms of pre-menstrual tension. There are other therapeutic benefits of chronic agonist treatment, such as control of precocious puberty¹⁸, and they may also be of value in treatment of breast and prostate cancer^{2,3}.

LHRH agonists also in principle permit partners to share the responsibility of fertility control. In a recent trial in men who self-administered daily injections of LHRH agonist for 6–10 weeks, sperm numbers fell markedly¹⁹ but, as with other methods that interfere with LH as well as FSH output, testosterone concentrations in the blood also declined, so the treatment was accompanied by loss of libido, impotence and even hot flushes. Thus, testosterone replacement is required and it remains to be seen how effective or practicable this would be. Another problem is that the doses which prevent ovula-

tion in women have no significant effect on the male reproductive function, precluding the use of nasal sprays. But, more efficient methods of delivery of LHRH analogues are expected — subcutaneous implants have already proved active⁵.

The mechanism whereby LHRH agonists exert this 'paradoxical' inhibitory effect remains a puzzle. It is believed that the pituitary is normally stimulated by pulses of LHRH from the hypothalamus to release pulses of LH into the peripheral circulation, which, together with FSH, control gonadal function. On this view, the agonists, some one hundred times more potent than endogenous LHRH, over-stimulate the pituitary with the result that the gonads are exposed to excessive gonadotrophin leading to the 'down regulation' phenomenon. Repeated exposure to agonists, on the other hand, reduces the pituitary's ability to release large amounts of gonadotrophin such as occurs at the pre-ovulatory surge^{13,20}.

This understanding has recently been

complicated by the recognition of direct gonadal actions of LHRH, involving functions which can both stimulate and inhibit steroidogenesis, and the finding of gonadal LHRH receptors.

As things are, LHRH analogues are not the answer to rapid population growth in developing countries because of the mode of administration, yet millions of women are seeking a better alternative to the 'pill'. Moreover, as Djerassi has pointed out²², while we fear the population explosion and demand better methods of contraception, the advent of the 'pill' has also led to an excessive fear of side effects, a suspicion of new developments and an environment in which pharmaceutical companies find it financially impossible to develop new contraceptives. LHRH agonists are not free from problems, but they work, and most important, to millions of women they should be more acceptable than the 'pill'. We should tackle their shortcomings because they are our brightest prospect for a new approach to contraception. □

The elixir of life

from an aging correspondent

ALEX COMFORT in his recent article *Gerontology in adolescence* (*Nature* 296, 179; 1982) declared that postponing diseases of the elderly beyond the normal life span was probably the area where most advance was likely in geriatric health. Nevertheless, he expressed the hope that an overall neuroendocrine timekeeper will be identified which may allow modulation of life span itself. It has long been a puzzle why some vertebrate species have long life spans — sulphur-crested cockatoos, for example, have been known to live for 130 years — whereas others are unlikely to survive two consecutive seasons. Moreover, long-lived poikilotherm vertebrates, such as giant tortoises and certain fish, do not stop growing with maturity, and it is these animals that show little, if any, evidence of senescence at the cellular or tissue level. New studies suggest that specific anti-senescence factors sustain the growth of these organisms.

The species which has received most attention from gerontologists is the freshwater carp, *Cyprinus carpa*. The carp has an ancient lineage of pisciculture — as a source of food in Roman and mediaeval Europe and as ornamental goldfish in the Orient. Their longevity is legendary and has recently been confirmed by carbon-dating. Dlouhý-Zivot and Ryba (*Acta gerontol. (Prag.)* 26, 143; 1981) analysed the ratio of ¹⁴C in the vertebrae and scales of two carp weighing over 30 kg taken from the fish pond of Gregor Mendel's former monastery near Brno; it was evident that

these same fish had been lurking in the muddy depths several years before Mendel first bred his peas only 100 m away. The European carp grows very slowly and it would appear that the long, cold winters aid its longevity. In Israel, where carp are an important source of protein, the fish grow very much faster, but attempts to keep long-lived carp as founder-breeders for commercial strains had not, until recently, been successful in the Galilean climate (Lechaim *et al. Israel J. Piscicult.* 12, 86; 1979).

The endocrine basis of growth and senescence in the carp has long been the focus of attention of Obispo and his colleagues in the Stoyte Institute of Life Sciences at Tarzana College, California. Boone and Obispo (*Am. J. Nutr.* 42, 1213; 1960) first investigated factors in the gut because of Hauberk's remarkable claim some 125 years earlier at the age of 92 (*Phil. Trans. R. Soc. B* 103, 397, 183; 1834) that addition of raw carp viscera to the diet each Friday prolonged fitness and sexual vigour. Obispo and Maunciple (*Am. J. Nutr.* 58, 82; 1967) extracted a protein factor from the intestine of carp which significantly extended the life span of gnotobiotic mice. After many a summer's hard work, detailed analysis revealed that the factor is a small protein, named longevin, comprising two dissimilar subunits, a hexadecapeptide A-chain and an icosapeptide B-chain linked by disulphide bonds (Maunciple *et al. J. molec. Endocr.* 2; 575; 1976). The activity of longevin is

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extremely heat labile on account of the instability of the B-chain. Three years ago Obispo's group made the notable discovery (Eldestez *et al. J. molec. Endocr.* 5, 902; 1979) that the B-chain was synthesized by *Escherichia coli* in the visceral flora of the carp whereas the A-chain was released from neuroendocrine Kultschitzky-like cells in the gut wall.

At last month's meeting of the International Gerontological Society in Athens, Obispo announced that the B-chain gene had been cloned and sequenced. Furthermore, by manipulating the sequence to code for lysine in place of arginine three residues from the C-terminus, a heat-stable molecule was obtained which still forms a functional complex with the A-chain. When bacteria expressing the modified B-chain gene in a mobilizable plasmid were introduced into 18 month-old specific-pathogen-free mice, prolongation of the life span was observed (*Proc. natn. Acad. Sci.*; in the press). Apparently the modified B-chain forms functional complexes in the mouse gut with a host hexadecapeptide homologous to that of the carp. Introduction of the B-gene into mice genetically predisposed to progeria did not result in delayed aging, suggesting that the host A-chain was deficient in these mice.

Some peculiar side effects were noted in mice colonized by bacteria producing longevin B-chain. The pelage on the body was replaced with scales as on the tail, and about 20 per cent of the mice developed multiple carcinoid tumours of the colon. These tumours release large amounts of longevin A-chain, as well as serotonin. A cDNA library has been prepared from the tumour cells and Obispo is confident that his laboratory has already identified the A-chain gene by co-transfection of the cDNA library together with the cloned B-chain gene into senescent MRC-5 cells. He is optimistic that the administration of a plasmid containing both A- and B-chain genes into the gut flora of mice will prolong life without inducing tumours, or hopefully the scaly skin. Although he has patented the B-chain clone, he admits that treatment of humans is still some years away.

The carp is unlikely to be the only source of longevin B-chain. Gerontologists are intrigued by the unusually long life spans of cold-water fishing communities as far apart as the Falkland Islanders and the Aleut Indians (Whiting *et al. WHO World Nutrition Rep.* No.5, 110; 1980), where raw krill is an important source of vitamins A, D and E. In northern Hokkaido, Furō and Chōju (*Jap. J. Publ. Hlth* 17, 646; 1975) reported exceptional vigour among the elderly fisherfolk who eat whole salted sprats. And the parish registry on Canna in the Inner Hebrides reveals several centenarians in the nineteenth century when kippers were smoked and eaten without evisceration (Gonister *J. comp. Nutr.* 29, 82; 1978). Long life is a fishy business indeed. □

Cancer genes — processed genes — jumping genes

from Peter Newmark

NEW data, some of it just published and some presented at a meeting* four weeks ago, illustrate the growing convergence of opinion on the activation of certain cellular genes (transforming genes) as the molecular basis of cancer. Whilst there is undoubtedly a danger of oversimplifying and generalizing from these studies, there is no doubt that they deserve attention, not least because of speculation that the genetic changes associated with cancer might be better understood in the light of our dramatically changed notions of the stability of the eukaryotic genome.

A variety of evidence strongly supports the view that, within the human genome, there are genes whose activation from dormancy or, at most, low-level expression is associated with the conversion of a normal cell into a tumour cell. Part of the evidence is the isolation, from human cells, of DNA sequences and RNA transcripts which cross-react with the transforming genes of tumour viruses of animals. But the most dramatic evidence of recent times is that it is possible, occasionally, to extract from human tumour cells, DNA that will transform cultured mouse fibroblasts into cells which, like tumour cells, form foci in culture and can induce tumours in mice. This DNA is said, therefore, to consist of, or to contain, a transforming gene. (It should not be overlooked, however, that these fibroblasts, NIH 3T3 cells, are not normal in all respects. Like tumour cells they are immortal in culture and they tend spontaneously to form foci in culture even before transfection with DNA from a tumour cell. What is more, they are the only type of cell that can reliably be transformed by human DNA.)

In Houston, M. Wigler (Cold Spring Harbor) outlined his group's progress (much of which appears on p.404 of this issue) in isolating and characterizing one transforming gene and R. Weinberg (Massachusetts Institute of Technology) described parallel studies with what is probably the identical gene from a different tumour. Each group started with a different human bladder carcinoma cell line and ended up with a very similar cloned fragment of DNA that retained the ability to transform NIH 3T3 cells. And both groups have used this fragment to detect similar sequences in the DNA of normal human tissues, implying that the transforming genes of tumours are neither the result of a major modification of normal genes (for example by rearrangement) nor the result of genetic import (for

example, by a tumour virus).

Further support for the lack of major modification comes from Weinberg's failure to detect any differences in the way eight restriction enzymes cut the transforming gene and its normal counterpart. Wigler, however, does find some small differences but there are as many between different tumour cell lines and between different normal human placentas as there are between tumours and normal tissues. This extensive polymorphism, Wigler speculates, is due to the presence of a variable number of copies of a short sequence of DNA in the environs of the transforming gene.

It is generally assumed that transforming genes are transcriptionally inactive in normal tissue and that cell transformation is a consequence of their activation, by whatever means. Not surprisingly therefore, Wigler finds transcription of the bladder transforming gene in the cell line from which it can be transferred to NIH 3T3 cells but not in normal tissue. More surprising was his claim in Houston that transcription of the bladder gene could be detected in every tumour cell line examined, including some which have a transforming gene different from that of the bladder cell line. Both Wigler and Weinberg have extracted at least three distinct transforming genes from human cell lines derived from different tissues but have found no definite correlation between the tissue and gene types (in addition to the bladder carcinoma gene, Wigler has also cloned the distinct transforming gene of a lung carcinoma cell line).

What is the basis of the transcriptional activation of the transforming genes? One rather unlikely possibility is that it is the result of amplification of the gene. There is evidence of amplification of mammalian transforming genes on an evolutionary time scale (Chattopadhyay *et al. Nature* 296, 361; 1982) but on a developmental time scale there are few examples of amplification (the most recent of which is the transient amplification of chicken actin genes during myogenesis — see W.E. Zimmer and R.J. Schwartz in *Gene Amplification* ed. Schimke, R.T.; Cold Spring Harbor Laboratory, New York; 1982). Both Weinberg and Wigler have explored the possibility of amplification in a panel of human carcinoma cell lines with their transforming gene probes. Weinberg finds no more than a twofold difference and Wigler at most a fivefold difference between different cells with his bladder gene probe and none with his lung gene

*'Perspectives on genes and the molecular biology of cancer'; the 35th Annual Symposium of Fundamental Cancer Research, held at Houston, Texas, on 2-5 March 1982.

probe. The evidence for gene amplification as a basis of transcription is therefore weak, particularly since even apparent amplification may in fact be the result of the presence of pseudogenes or of abnormal numbers of chromosomes.

It is much more likely that transcriptional amplification is the result of switching the transforming gene on (or up) by, for example, a mutation or insertion in a site that regulates transcription. In theory that could be revealed in a matter of months when the sequences of the transforming gene from bladder tumour cells and normal cells will be available for comparison but it may be difficult immediately to prove that any change of sequence is responsible for increased transcription.

Such is the situation in bursal lymphomas of chickens. There is good evidence that these are caused by the integration of avian leukosis virus (which does not carry a transforming gene) into the host genome in such a way that a cellular transforming gene comes under the influence of a viral promoter. The problem arises from the fact that the inserted promoter need not be in the expected position — just upstream of the transforming gene — but may also be downstream or even in the 'wrong' orientation (*Nature* 295, 209; 1982). By way of indicating that this well documented system is not unique, Harold Varmus (University of California, San Francisco) presented evidence of the localization of murine mammary tumour virus sequences within the same 6 kilobase fragment of the genome of 5 out of 35 mammary tumours caused by the virus. This localization is reminiscent of, if less frequent than, that of avian leukosis virus sequences in bursal lymphomas. So far, however, Varmus has no evidence that the 6 kilobase site contains a transforming gene or produces any transcripts.

The cellular transforming gene which is activated by avian leukosis virus is one of a class of such genes that are defined by their relationship to the transforming genes carried by many acute tumour retroviruses. (So far there is no evidence, but some expectation, that these cellular transforming genes, and those exemplified by Wigler and Weintraub, are of the same class.) It is assumed that viral transforming genes are derived from the mRNAs of cellular transforming genes. This assumption fits with the fact that both the viral genes and mRNAs lack the introns of the cellular genes. Howard Temin (University of Wisconsin-Madison) described such a case in which the *rel* oncogene of reticuloendothelial virus (strain T) has clearly been derived from the mRNA of a cellular oncogene that is found in birds (but not mammals). The cellular gene occupies a very big stretch of DNA because of its introns and in the turkey, the gene is highly polymorphic, perhaps because of deletion/insertion events in the introns.

The discovery of what Philip Leder (NIH and Harvard Medical School)

dubbed 'processed genes' strongly suggests that transforming genes are not the only cellular genes that can be re-integrated into chromosomal DNA. Like viral transforming genes, processed genes have a sequence closely resembling the coding sequence of a cellular gene. What was probably the best case of a processed gene, that of α -globin (Lueders *et al.* *Nature* 295, 426; 1982), has now been superseded by the case of human immunoglobulin λ light chain (Hollis *et al.* *Nature* 296, 321; 1982). This processed gene terminates in a long sequence of adenosines, like the 'poly(A)' tail of the mRNA from which it must surely have been derived by reverse transcription and integration. (Note, however, that the mRNA in question appears to have been derived from an incomplete, abnormal transcript.)

But the most striking processed gene will be that of human β -tubulin (Wilde *et al.* *Nature*; in the press). It appears to have been derived from a complete transcript. It lacks introns and has a poly(A) tail. And, it is bounded by a directly repeated 11 base pair sequence. This reminds one of the sequences that flank all transposed genes and make it yet more likely that the gene was integrated as supposed.

Another hallmark of a processed gene is

that it can be located far from the parental gene, even on a different chromosome. M. Birnstiel (University of Zurich) raised the possibility that a gene could pass not just between chromosomes but between species. In analysing the histone genes of various species of sea urchin, his group has come to the startling conclusion that within the last million years, a cluster of histone genes jumped from one species to a distantly related species with which it lives in the Atlantic Ocean. How else is one to explain the fact that there is little difference between the third base positions of one histone gene cluster of the two species whereas the difference between other clusters in these and other species is always reasonably great and consistent with a fixed rate of change with evolutionary time (M. Busslinger *et al.* *The EMBO J.* 1, 27; 1982). Was this also a virally mediated jump?

In several ways, therefore, loose but tantalizing threads hold together the genetic mechanisms of cancer and evolution. The links may be illusory but even if they vanish with time, it is more than likely that others will be forged as molecular biologists continue, in Leder's modest description, "to stumble" across new varieties of genomic fluidity. □

Geomagnetic cores and secular effects

from D.H. Tarling

ALTHOUGH the geomagnetic field was the subject of the earliest scientific treatise (Gilbert's *De Magnete* of 1600) it still remains the most enigmatic of all the Earth's properties. The situation is even more extraordinary when it is realized that it is also the only geophysical property whose value can be determined for past times by direct measurements of rocks at the Earth's surface. Although the lack of progress reflects, in part, the complexity of the magnetohydrodynamic equations that govern core processes, the chief problem — given the number of geomagnetic models available for testing — must be seen as a lack of data.

The problem has been partially resolved in this century by the establishment of geomagnetic observatories and by the increasing number of field studies, supplemented by satellite monitoring. The present geomagnetic field is now quite well known, but the long-term changes in the field, recorded in rocks, are not — they have mainly been studied for other purposes, particularly geological and archaeomagnetic dating. A recent meeting at the Royal Society of London* was thus significant in

concentrating a variety of minds on defining magnetic data in terms of core processes and complementing seismic, geochemical and geomagnetic data with theoretical considerations.

There are, in fact, numerous observations of the geomagnetic direction, especially declination (and to a lesser extent intensity), available from observations in the 18th and 19th centuries. Thompson (University of Edinburgh) and Malin (Institute of Geological Sciences, Edinburgh) have re-examined much of these data with somewhat surprising results. It seems that the lifetime of the components of the non-dipole field (isoporic foci) decay in much shorter periods, some 500 years, than had been previously thought. The present westward drift of the field has been occurring on a world scale for most of the past 400 years and a change in the sign of the quadrupole component occurred in 1820. Studies of lake sediments (Creer, University of Edinburgh; Barton, University of Rhode Island) and archaeological materials (Kovacheva, Bulgarian Academy of Science; DuBois, University of Arizona) show clearly that intervals of both eastward

*The meeting entitled "The Earth's Core: Its Structure, Evolution and Magnetic Field" was held on 27–28 January 1982.

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and westward drift have occurred. Creer found evidence for both correlations and lack of correlations in lake sediments from Europe and North America which Thompson explained as the simple growth and decay of non-dipole features — stationary components arising where two non-dipole components of the same sign are interacting. The presence of both clockwise and anticlockwise rotations over archaeological periods means that the westward drift cannot be adequately explained by a persistent faster rotation of the mantle relative to the core. However, the rate of secular variation and the rate of rotation of the Earth are clearly coupled, as indicated by the 1969 'jump' in secular variation (Barracough, Institute of Geological Sciences, Edinburgh), but the lunar tidal deceleration of the mantle of 1.78 ms per century may be accompanied by a non-tidal acceleration of 0.8 ms per century (Runcorn, University of Newcastle) attributable to a continued but very slow growth of the core, as also suggested on geochemical grounds (Allegre, University of Paris).

All geomagnetic models are constrained by the need to allow polarity reversals. It is generally agreed that reversals occurred randomly during over the past 48 million years but may have occurred more systematically before that. However, it is probable earlier reversals have simply not been dated with sufficient precision. Lowrie (University of Zurich) and Channell (Lamont-Doherty Geological Observatory) presented improved biostratigraphical dating of reversals in the Mesozoic that are vital for establishing world-wide correlation scales. Unfortunately, analyses of the geomagnetic polarity frequency still require conversion of the biostratigraphical time scales into an accepted radiometric ('absolute') scale. The new radiometric decay constants for K/Ar have been designed to give a greater uniformity in radiometric ages determined by different methods (Steiger & Jager *Earth planet. Sci. Lett.* 36, 359; 1977), but consistent errors of several per cent can still arise in establishing the radiometric age of biostratigraphical horizons (Tarling & Mitchell *Geology* 4, 133; 1976). Careful analyses and assessments are therefore necessary before the polarity frequency in the early Tertiary and Mesozoic can be considered to be established.

Polarity transitions are likely to provide key diagnostic information on the processes operating in the geomagnetic dynamo but have proved very difficult to study. Thin slices of oceanic sediment cores can be used to determine more precisely the changes at specific locations along a line of longitude (Opdyke, University of Miami), but observations are mostly confined to the last geomagnetic transition and to the Northern Hemisphere (Hoffman, California Polytechnic). Sediments can, in any case, be disturbed by non-geomagnetic effects and Verosub (University of Cali-

fornia, Davis) remains rightly unconvinced about much of the available sedimentary evidence for most polarity excursions, even though some undoubtedly exists. Even records in igneous rocks may be dominated by rock magnetic factors, such as in the Laschamp-Olby excursion (Heller, University of Zurich), and studies of igneous sequences, without adequate radiometric control, can also be misleading. It is now clear that the 'dipole window' in the Pacific did not exist over the past few million years or so, but is simply an artefact of the very rapid eruption rates in Hawaii (McWilliams, University of Stanford), although the corresponding palaeomagnetic poles continue to be slightly 'far-sided' and right-handed, as in Iceland, posing problems in any interpretation of the average geomagnetic field (Harrison, University of Miami).

The geomagnetic dynamo does not require a large driving energy and sufficient could be provided by the solidification of the inner core. The outer core is probably enriched in carbon, sulphur and oxygen (Ahrens, Caltech), so it is likely that further energy is provided by the incorporation of some radiogenic heat-producing elements, such as K, Th and possibly U. The core has generally been neglected in considering the distribution of lead and anomalies found in rocks derived from the mantle have often been interpreted as showing that mantle convection could not take place. Allegre considered that the core could, in fact, be a major factor in the continuing evolution of the core and mantle.

The evidence for actual motions in the core was somewhat controversial, with LeMouél (University of Paris) considering that there are clear signs, in geomagnetic measurements, for such motions, while these were not seen by Gubbins (University of Cambridge). The most provocative point concerned whether or not there are 'bumps' on the core-mantle interface. These are now more closely defined by the seismic evidence (Bolt, University of California, Berkeley), particularly when observed signals are compared in both amplitude and shape with theoretical seismograms. The 'scattering structures' appear confined very closely to the core-mantle boundary with lengths some 10–30 km and heights of a few 100 m — if they are topographical 'bumps' (Haddon, Bureau of Mineral Resources, Ottawa). However, they could equally well mark zones of temperature or physical differences rather than topographical boundaries. Either explanation may, of course, also be linked to the convective pattern in the mantle (Hide, Meteorological Office, Bracknell). The presence of numerous bumps may cause an increased amplitude in secular variation and also an increased probability of reversals of polarity, and quieter magnetic periods, on a geological time scale, may be associated with fewer bumps. This demonstrates, yet again, the need for closer examination of the palaeomagnetic records to see whether there really is any correlation between the magnitudes of secular variation and the frequency of polarity transitions. □

A galaxy for quasar 3C48

from R.F. Carswell

THE superb observations by Boroson and Oke reported in this issue of *Nature* (see p.397) reveal that the faint nebulosity to the north and south of the quasar 3C48 is dominated by starlight and that the stars have a redshift close to that determined from the broad emission lines in the quasar spectrum. This provides the first direct evidence that a quasar is associated with a galaxy and lies at a distance corresponding to its redshift, adding to the weight of evidence against non-cosmological quasar redshifts.

Most quasars are so far away that there is little hope of detecting galaxies which might be associated with them, but for a number of low-redshift examples, faint surrounding nebulosity has been found. The difficulty has then been to establish that this extended emission arises from starlight and not from a large gaseous envelope which radiates only in emission

lines. A number of attempts have been made to do this, by obtaining spectra of the nebulosity to search for the stellar absorption lines typical of galaxies, but until now the results have been somewhat unconvincing.

While Boroson and Oke's work confirms a few widely held beliefs, the results also contain surprises. The first, and most obvious, is the discovery that the stars in the 3C48 galaxy are not quite like those in a normal galaxy such as our own. They appear to be bluer, and younger, than usual, similar to those in galaxies where recent bursts of star formation are thought to have occurred. Estimates of age are difficult, but the brightest part of the stellar population in 3C48 is probably 100 million to a billion years old. If the stars have formed recently we would expect the system to be rich in gas, and certainly the material presented by Boroson and Oke shows, through emission lines in the spectra of the nebulosity, that there is still much gas present. The galaxy could thus be

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a gas-rich spiral rather than an elliptical which has little interstellar material, though given the distance of 3C48 it will be very difficult to confirm the presence of spiral structure.

It is also tempting to suggest that those quasars with strong emission lines are formed in spiral (that is, gas-rich) galaxies. In all cases, only very small amounts of gas are needed very close to the quasar to explain the line strengths seen, but then only a small amount of the available gas will be in the immediate quasar neighbourhood. BL Lac objects, which are really quasars with no emission lines and therefore probably little gas in their neighbourhood, could similarly be in the centres of elliptical galaxies (which have a low gas abundance). The late-type stellar spectrum recently found for the nebosity around BL Lac is itself suggestive in this regard. If young stars do generally dominate the quasar stellar spectrum, this incidentally helps to

explain why previous searches for starlight have failed. Most of these have looked primarily for the usual strong Ca II feature typical of a later population, which is weak or absent in the 3C48 spectrum. But much work remains to be done before this can be established; one object, however important, is hardly a statistical sample!

Another interesting, and more puzzling, result is that the redshift of the quasar determined from the galaxy starlight does not agree very well with that determined from the sharper emission lines in the quasar spectrum. While it is not unusual for the different broad emission lines to give rather different redshifts, the sharp lines are believed to arise in a more extended region and so to give a better redshift estimate. Here the sharp lines differ from the galaxy redshift by about 400 km s⁻¹ while the broad hydrogen lines agree very well. It is possible that a combination

of velocity flow and obscuration of the clouds shifts the sharp lines significantly, but the required shift is so large (comparable with the line widths) that it is rather difficult to explain simply.

Another surprise is simply one of observational detail. The spectra Boroson and Oke obtained for two regions of the nebosity around 3C48 took a total of only two hours of telescope time, while others in the past have unsuccessfully devoted much longer periods of time to similar projects. A combination of an efficient spectrograph and a modern charge-coupled device as a detector has allowed them to obtain useful spectra of faint objects quickly. With such a system available one hopes, and expects, that similarly exciting discoveries will continue to be made at Palomar, and that Boroson and Oke will be able to follow up their observations by examining the nebosity around other quasars. □



100 years ago

THE WINGS OF PTERODACTYLES

The first Pterosaurians discovered were recognised as flying animals, but were thought to be bats. As soon as their general structure became known, they were classed with the reptiles, although it was considered possible that their power of flight was due to feathers. Later their bones were mistaken for those of birds by various experienced anatomists, and others regarded them as sharing important characters with that group. Some anatomists, however, believed that the fore-limbs of pterodactyles were used for swimming rather than for flight, and this view has found supporters within the present decade. A single

fortunate discovery, made a few years since, has done much to settle the question as to the wings of Pterodactyles, as well as their mode of flight, and it is the aim of the present article to place on record some of the more important facts thus brought to light.

The specimen to be described was found in 1873, near Eichstätt, Bavaria, in the same lithographic slates that have yielded *Archaeopteryx*, *Compsognathus*, and so many other Jurassic fossils known to fame. This specimen, which represents a new species of the genus *Rhamphorhynchus*, is in a remarkable state of preservation. The bones of the skeleton are nearly all in position, and those of both wings show very perfect impressions of *volant membranes* still attached to them. Moreover, the extremity of the long tail supported a separate vertical membrane, which was evidently used as a rudder in flight. These peculiar features are well shown in Fig. 1, which represents the fossil one-fourth the natural size.

A careful examination of this fossil shows that the patagium of the wings was a thin smooth membrane, very similar to that of modern bats. As the wings were partially folded at the time of entombment, the volant membranes were naturally contracted into folds and the surface was also marked by delicate striae. At first sight, these striae might



Fig. 3 Restoration of *Rhamphorhynchus phyllurus*.

readily be mistaken for a thin coating of hair, but on closer investigation they are seen to be minute wrinkles in the surface of the membranes, the under-side of which is exposed. The wing membranes appear to have been attached in front along the entire length of the arm, and out to the end of the elongated wing finger. From this point the outer margin curved inward and backward, to the hind foot.

The membrane evidently extended from the hind foot to near the base of the tail, but the exact outline of this portion cannot at present be determined. It was probably not far from the position assigned it in the restoration attempted in the cut given below, Fig. 3. The attachment of the inner margin of the membrane to the body was doubtless similar to that seen in bats and flying squirrels.

In front of the arm there was likewise a fold of the skin extending probably from near the shoulder to the wrist, as indicated in Fig. 3.

The present species appears to be most nearly related to *Rhamphorhynchus Gemmingi*, von Meyer, from the same geological horizon, and near the same locality. That it is quite distinct, however, is shown, aside from the difference in size, by the complete ankylosis of the scapula and coracoid, and by the fifth digit of the hind foot being well developed, and having three phalanges. In the name *Rhamphorhynchus phyllurus*, here proposed for the species, the latter designation refers to the leaf-shaped caudal appendage, which appears to be one of its most characteristic features.

For the long delay in the description of this important European specimen, the writer can only plead *l'embarras des richesses* nearer home.

O.C. Marsh

Yale College, New Haven, March 14.

From *Nature* 25, 531, 6 April 1882.



ARTICLES

Detection of the underlying galaxy in the QSO 3C48

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Spectra have been obtained of the faint nebosity north and south of the centre of the QSO 3C48. In addition to the emission lines previously known, a continuum dominated by hot stars is seen at both positions. This suggests that the host galaxy is a spiral and may explain why searches for features indicative of an old stellar population in QSOs have been unsuccessful. The redshift of the underlying galaxy is the same as that of the permitted lines in the QSO but differs from the redshift of the forbidden lines in the QSO by 500 km s⁻¹.

THE QSO 3C48 has long been known to be associated with wisps of nebosity. These extend predominantly north and south from the central object and have been well illustrated by Sandage and Miller¹. Although it has been argued that QSOs occur in the nuclei of galaxies and that the underlying galaxy should be visible in some cases², this identification of the nebosity in 3C48 has been questioned for two reasons. First, its radial extent and total luminosity are uncharacteristically large for a normal galaxy³. Second, the spectrum of the fuzz has been obtained by Wampler *et al.*⁴ who found no evidence for starlight. They did detect emission lines from [O II], [O III], and [Ne III] at a slightly larger redshift (0.370) than that of the same lines in the QSO (0.368). Subsequently, Bergeron⁵ modelled the spectrum of the nebosity with an extended gaseous disk, ionized by the QSO.

Observations

We observed 3C48 and the surrounding nebosity on two nights, 4 November and 16 December 1981 with the Hale 5-m telescope. Instrumentation consisted of the double spectrograph with an RCA 320 × 512 CCD on the blue camera and a Texas Instruments 800 × 800 CCD on the red camera. The blue camera exposures were of poor quality and will not be discussed. The grating was set to give coverage from λ 5,000 to λ 10,000 with the red camera, yielding about 6 Å per pixel. A slit 2 arc s wide, orientated in the east-west direction was used in all exposures.

On 4 November 1981, we obtained a 500-s exposure of the nucleus and a 3,000-s exposure centred 2 arc s north of the nucleus of 3C48 in 2 arc s seeing. On 16 December 1981, we obtained an additional 500-s exposure of the nucleus and a 3,000-s exposure with the slit centred 2 arc s south of the nucleus. The seeing on the second night was 1 arc s. Comparison lamp spectra were obtained on each night immediately after each pair of exposures of 3C48.

The data were reduced by first correcting the two-dimensional image for bias level and small scale sensitivity variations. The sky spectrum was then determined and subtracted and a 4 arc s wide swathe was extracted to create a one-dimensional spectrum. The comparison lamp spectra were similarly extracted and fit with a cubic wavelength polynomial. Residual errors from these fits were <0.20 Å for each of nine comparison lines. Finally, the nuclear and nebosity spectra were corrected approximately to absolute fluxes using observations of spectrophotometric standards obtained on each night. All observations were made within 15° of the zenith so atmospheric refraction effects are negligible.

Figure 1 shows the nuclear spectrum obtained on the first night and the spectra of the two regions in the nebosity. The strongest lines in the nucleus have been labelled. The spectrum

of the north nebosity is contaminated by scattered light from the nucleus. This can be seen from the broad H β and H α and from the Fe II complexes. Attempts to remove this contamination by subtracting a fraction of the nuclear spectrum from the north nebosity spectrum showed that this technique is incapable of removing the scattered light at all wavelengths. Because many of the strong features expected in the integrated light from stellar populations are between λ 3,900 and λ 6,000 we limited our efforts to this spectral region. We found that the subtraction of 1.4% of the nuclear spectrum from the north nebosity removes all traces of the broad nuclear lines except for H α . This corrected north-nebosity spectrum and the uncorrected south-nebosity spectrum, which appears essentially free of scattered light from the nucleus, are shown in Fig. 2. For ease of identification of features, the spectra are plotted against rest wavelength, assuming a redshift of 0.370. We have not plotted the spectral region longward of λ 5,500 where the data become quite noisy and the residual contamination by the nuclear scattered light may be considerable.

Results

Figure 2 shows that in addition to the emission lines seen by Wampler *et al.*⁴ a continuum with absorption features is present in both north and south spectra. These absorption features consist primarily of the higher Balmer series lines and the Ca II K line at λ 3,933. H β appears to be completely filled in by emission and H γ partially so. None of the absorption features indicative of late type stars, particularly the G band (λ 4,300), Mg b (λ 5,175), or the Na D lines (λ 5,890), are seen. The [O I]

Table 1 Measured redshifts of 3C48

Line	Nucleus		Nebosity north	Nebosity south
	Run 1	Run 2		
[O II] λ 3,727	0.3688		0.3716	
[Ne III] λ 3,869	0.3686	0.3686		
[O III] λ 4,959	0.3680	0.3685	0.3707	0.3708
[O III] λ 5,007	0.3683	0.3688	0.3706	0.3700
H γ emitted	0.3702	0.3705		
H β emitted	0.3695	0.3698		
Ca II λ 3,933 absorbed			0.3713	0.3697
He absorbed			0.3704	0.3701
H δ absorbed			0.3721	0.3707
Means*				
Forbidden lines	0.3685 $\pm$ 0.0001		0.3710	0.3704
Permitted lines	0.3700 $\pm$ 0.0002		0.3713	0.3702
All lines			0.3711	0.3703
			$\pm$ 0.0003	$\pm$ 0.0002

* Means for nucleus are average of both runs.

$\lambda 6,300$ line, which is outside the spectral limits of Fig. 2 is also present in the nebulosity. It can be seen in Fig. 1 to be much stronger in the north-nebulosity spectrum than in the nuclear spectrum. The nebulosity apparently shows the spectrum of a hot stellar population, with a mean spectral type of about A7 as judged from the relative strengths of H ϵ and Ca II K and from the strengths of the higher Balmer lines. The continuum slope is also indicative of blue stars. The approximate $B - V$ colours are 0.35 for the north nebulosity and 0.22 for the south nebulosity. These estimates are probably only accurate to ± 0.1 mag.

It was pointed out by Wampler *et al.*⁴ that the forbidden lines in the fuzz have a slightly higher redshift than those in the nucleus. Similarly, Thuan *et al.*⁶ found a discrepancy between the redshifts of permitted and forbidden lines in the nucleus. Therefore, we have measured the redshifts of all reasonably strong and sharp lines in the nucleus and in the nebulosity: these are listed in Table 1. All the lines in each nebulosity spectrum have the same redshift. Both of these redshifts are consistent with the redshift of the permitted lines in the nucleus. The forbidden lines in the nucleus, however, have a velocity which is, in the rest frame of the QSO ($\Delta z/(1+z)$), $330 \pm 78 \text{ km s}^{-1}$ less than that of the permitted lines and $481 \pm$

106 km s^{-1} less than the mean of the redshifts of the two regions in the fuzz. The uncertainties quoted are derived from the mean errors in averaging the redshift determinations from the various lines.

Discussion

Several recent studies have used imaging techniques to produce evidence for extended structure in low redshift QSOs⁷⁻⁹. The identification of this fuzz with galaxies, however, rests on circumstantial evidence. The few objects in which the nebulosity has been observed spectroscopically have shown only emission lines, that is, no evidence of a continuum or absorption features indicative of stars^{4,9-11}. The one possible exception to this is 3C206; the fuzz around it shows a red continuum with Ca II H and K absorption¹². However, other absorption lines expected to be strong are absent. Also, attempts to show the connection with galaxies by observing the highly diluted stellar features in the nuclei of QSOs have been unsuccessful¹³, unlike similar studies of N galaxies and BL Lac objects^{14,15}. Thus, the discovery of unambiguous stellar absorption features in the fuzz around 3C48, a fairly luminous QSO ($M_V = -25.3$ for $q_0 = 1/2$) is a crucial link in relating QSOs to active galactic nuclei and to normal galaxies.

The immediate goal of identifying the fuzz around QSOs is to ascertain the properties of the regions in which QSOs form. In that regard the early spectral type of the underlying galaxy is unexpected. In general, of course, blue colours and recent star formation are indicative of spiral galaxies as opposed to ellipticals. However, there are elliptical galaxies with young stars, NGC1275, for example¹⁶. Also, the average $B - V$ colour of the fuzz, 0.28 is bluer than a typical galaxy of even the latest type¹⁷. The mean $B - V$ colour for Sc galaxies is 0.51 (ref. 17), more than 0.2 mag redder than is observed in the fuzz of 3C48. The presence of gas required to explain the emission lines also argues against the host galaxy being an elliptical as these usually have little or no detectable gas¹⁸.

The surprisingly blue spectrum of this nebulosity raises the question of whether this is a common phenomenon in galaxies in which QSOs reside, perhaps because shocks emanating from the central source trigger global bursts of star formation. It is clear that the spectrum is really dominated by the hot stars as there is no evidence for a cooler population even at Mg I b $\lambda 5,175$ or Na D $\lambda 5,890$ where the old population should contribute a larger percentage of the integrated light than in the blue. The single burst models of stellar populations calculated by Larson and Tinsley¹⁹ have $B - V$ colours in the observed range after $1-2 \times 10^8$ yr. However, the mean spectral type of A7 at $\lambda 4,000$ argues for an age more likely 10^9 yr. This discrepancy, if real, may indicate a very flat initial mass function which would produce a slower reddening in $B - V$ with age¹⁹.

Another aspect of the extreme youth of the stellar population is the total luminosity of the underlying galaxy. It has been suggested in previous work that the nebulosity is too luminous to be a galaxy³. We estimate that the total apparent magnitude is 18.5 or brighter at $\lambda 5,500$ in the rest frame of the galaxy; this corresponds to an absolute visual magnitude of -23.0 ($H_0 = 50$, $q_0 = 1/2$). While this is particularly luminous for a normal spiral or elliptical galaxy, the blue colour corresponds to a population with a mass-to-light ratio, M/L_V , at least a factor of 10 smaller than those typical of older populations¹⁹. Thus, this galaxy could have become as much as 3 mag brighter because of the presumed burst of star formation. If this is not unusual, that is, if QSOs frequently occur in gas rich galaxies in which they trigger global episodes of star formation, the technique of searching for weak stellar absorption features in the nuclei of QSOs may be futile.

Wampler *et al.*⁴ observed the north nebulosity at twice the distance from the nucleus of the observations described here. They found no evidence for the stellar continuum at the more distant position, but did find emission lines from forbidden

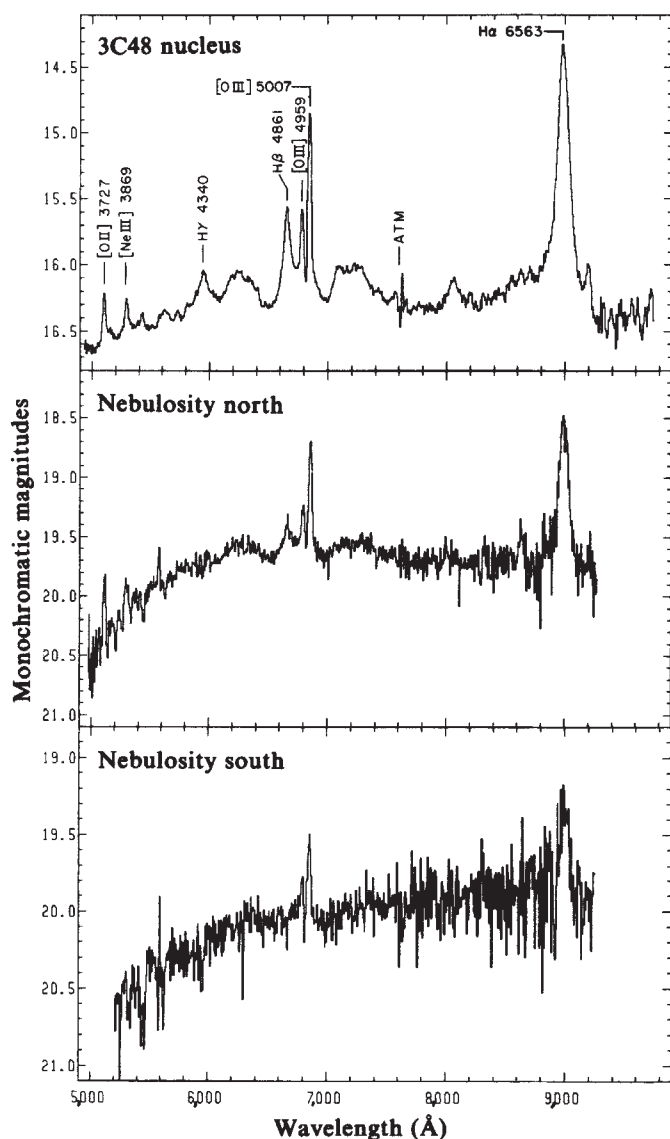


Fig. 1 Spectra of the nucleus of 3C48 and two regions in the nebulosity. The strongest spectral features in the nucleus are labelled. The feature ATM is due to the atmospheric A band.

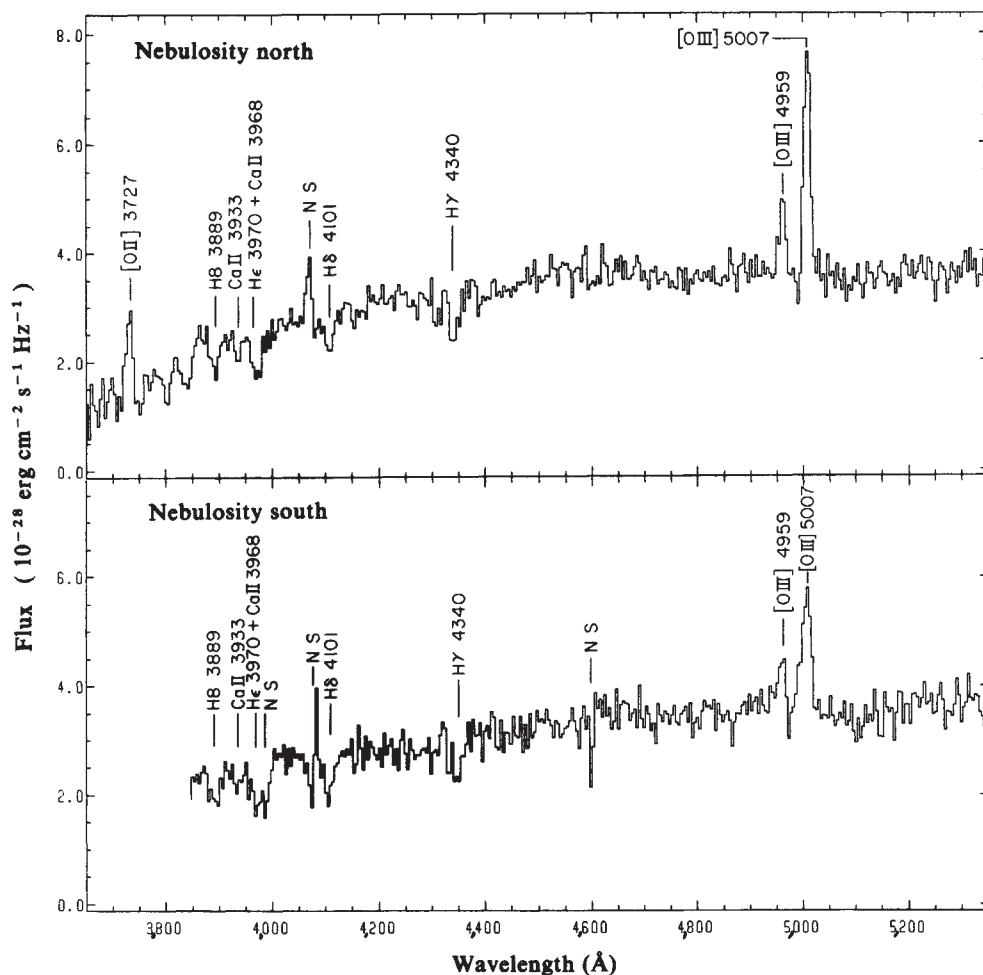


Fig. 2 Spectra of the underlying galaxy in 3C48 plotted against rest wavelength. The spectrum of the northern region has been corrected as described in the text. Prominent features have been identified. The features NS are due to poor subtraction of strong night sky lines.

transitions of O II, O III, and Ne III. They and later Bergeron⁵ argued that the emitting gas was ionized by the QSO. Bergeron's detailed analysis had trouble explaining the absence of H β emission which seemed to rule out heating by a Lyman continuum source. In our spectra, it is clear that the apparent absence of H β emission is due to the presence of H β absorption in the underlying continuum and also that the intrinsic strength of the H β emission is approximately the same as the [O III] λ 4,959 line. In support of the idea that the ionization is due to a power law continuum, we note the strong presence of three levels of ionization of oxygen; we have not, however, performed a detailed analysis of the line strengths.

One of the peculiar aspects of the fuzz is the redshift discrepancy. Table 1 shows that the forbidden lines in the QSO are 300–500 km s⁻¹ blueshifted from both the permitted lines in the QSO and all of the lines in the underlying galaxy. Presumably, then, it is these forbidden lines in the QSO which differ from the systemic redshift. This phenomenon has been seen in Seyfert I systems and QSOs previously^{6,20} but it has never been clear which lines, the forbidden or the permitted, represent the systemic velocity. Explanations proposed for this discrepancy include models with infalling or outflowing gas in

which one side of the region is preferentially seen because of dust mixed with the gas or light travel time arguments^{20–22}.

Conclusion

We have detected stellar absorption lines in the nebulosity around the QSO 3C48. The integrated light from this population has both a $B - V$ colour and absorption features indicative of middle to late A stars, suggesting that the underlying galaxy is a spiral in which a massive star formation burst was triggered at least 10⁸ years ago. The [O II] λ 3,727 and [O III] λ 4,959 and λ 5,007 emission lines seen by Wampler *et al.* as well as [O I] λ 6,300 are also present. The underlying galaxy must have an absolute magnitude of -23 , or brighter but without the recent burst of star formation it would be at least 2 mag fainter. The redshifts of both absorption and emission lines in the fuzz are inconsistent with the forbidden lines in the nucleus but, within measurement errors, are the same as that of the permitted lines. This indicates that the nuclear forbidden lines are blueshifted 300–500 km s⁻¹ with respect to the systemic velocity.

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Late Cenozoic uplift of stable continents in a reference frame fixed to South America

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A reference frame fixed to South America may approximate to an 'absolute' reference frame for the past 100 Myr. In this frame, otherwise stable continents that have moved onto the former positions of oceanic ridges have experienced rapid plateau uplift, probably caused by phase changes in the continental mantle, and occasional volcanism with time lags of up to 70 Myr.

ALTHOUGH plate tectonics accounts quantitatively for many geological phenomena, it does not explain—even qualitatively—large-scale continental uplift in otherwise stable areas. Migration of continents over 'hotspots' may account for uplift of continental areas that are as much as 1,000 km across¹, but is not applicable to areas such as southern Africa where hotspots of the appropriate dimensions seem to be absent. Yet heating is the only process that could bring about the necessary uplift. Heating can cause expansion as in the oceans², or it can induce a phase change^{3,4}, which will also be accompanied by thermal expansion. Quantitative continental uplift models attribute uplift to some form of thermal anomaly in the continental asthenosphere⁵. Given a thermal anomaly, its passage through the continental lithosphere to the surface may cause effects that correspond closely to field observations, but possible causes of the anomaly—chemical and/or thermal mantle 'plumes', convection or shear heating—cannot yet be discriminated from one another.

The hypothesis advanced here is that a small amount of heat has been put into the base of the continental lithosphere where the past positions of ocean ridges younger than ~100 Myr, relative to South America, now lie under continents. I demonstrate that two such areas—'high Africa', and south-east Australia—have experienced strong plateau uplift some 50–60 Myr later, whereas adjacent continental areas, which lie just beyond these former ridge positions, have not. Moreover, in south-east Australia the pattern of Cenozoic volcanism lags behind in a remarkably systematic way the apparent traces of newly formed ocean floor under that continent.

Late Cenozoic uplift of Afro-Arabia

Africa is the continental part of a plate that includes much of the south-east Atlantic Ocean and the south-east Indian Ocean. Plate margins lie close to Africa only on its northern and north-east margin. A nascent plate margin may run along part of the African rift system.

The African continent has undergone several uplift cycles⁶, the most recent of which is the Plio-Pleistocene uplift ('cymatogeny') that has created the present division of Africa into 'high Africa' and 'low Africa'⁶ (Fig. 1a). Significant uplift may have started as early as Miocene time (C. Hartnady, personal communication). Before the late Cenozoic, most of Africa was a continent of low relief, with an "elevation above sea level rarely more than 600 m"⁶. The Plio-Pleistocene cycle of uplift raised most of high Africa by ~1,200–1,500 m (ref. 6).

As might be expected, high Africa is associated with negative Bouguer gravity anomalies^{7,8}, whose source is attributed to variations in the level of a boundary between a deeper low density layer and an overlying high density layer in the upper mantle. This boundary can be regarded as the base of the lithosphere.

The lithosphere can also be defined by combining Bouguer anomalies with teleseismic delay times⁸, or by heat flow models⁹. Though differing in detail, all models agree that high Africa is underlain by generally thinner lithosphere than is low Africa. Thickness estimates vary considerably for low Africa: from about 250 (ref. 5), 225 (ref. 8) to 100 (ref. 7) km, with similar variations for high Africa. Recent work suggests that 'normal' continental lithosphere may be no thicker than oceanic lithosphere⁹ that is 100 km. As a compromise, I take the lithosphere under low Africa today to be ~150 km thick, its inferred Cretaceous thickness under high Africa¹⁰.

Uplift of the continent due to the physical expansion of the lithosphere should also affect the adjacent ocean floor because the boundary between high and low Africa reaches the coast. Inspection of the ocean floor bathymetry¹¹ reveals no substantial topographic anomaly across the Atlantic seaward extension of this boundary, though the effect may be negligible. Thus the topographic anomaly is largely restricted to the continent and does not appear to have its origin mainly in the thermal expansion of hot rock. This conclusion is supported by thermal calculations.

Table 1 Distance in degrees of global mean palaeomagnetic pole from geographical pole (figures not in parentheses) and distance travelled in time interval (figures in parentheses)

Continent	20	20–40	40	40–60	60	60–80	80	80–100	100 Myr
Antarctica	5.7	(1.6)	4.5	(8.1)	6.1	(3.1)	3.6	(11.7)	10.2
Africa	8.2	(2.1)	9.6	(7.8)	14.2	(6.0)	20.2	(4.6)	23.8
Eurasia	6.5	(1.0)	7.2	(2.7)	8.2	(2.6)	10.3	(4.2)	11.8
North America	7.8	(1.8)	9.6	(4.3)	11.9	(5.3)	11.1	(1.0)	12.0
South America	6.0	(2.7)	5.6	(4.0)	3.3	(2.8)	6.1	(5.6)	3.2
No. of poles	89		60		72		46		52
α_{95}	2.5		4.4		4.3		4.7		4.7

The 20 and 40 Myr data are from ref. 22; the 60, 80 and 100 Myr values use the same data base but exclude all Indian poles because of the problem of weighting poles from a rapidly moving fragment. Distances are the distance in degrees of the global mean palaeomagnetic pole calculated for the named continent from the geographical pole. The distance travelled by the pole in successive 20-Myr intervals has been calculated from successive pole positions. These can be non-zero even if the mean pole has remained at a fixed distance from the geographical pole. The global mean pole averages all known palaeomagnetic data up to 1979 from stable continental fragments on appropriate continental reconstructions²². Poles for India and Australia have been omitted because these continents move substantially with respect to the poles in this time interval.

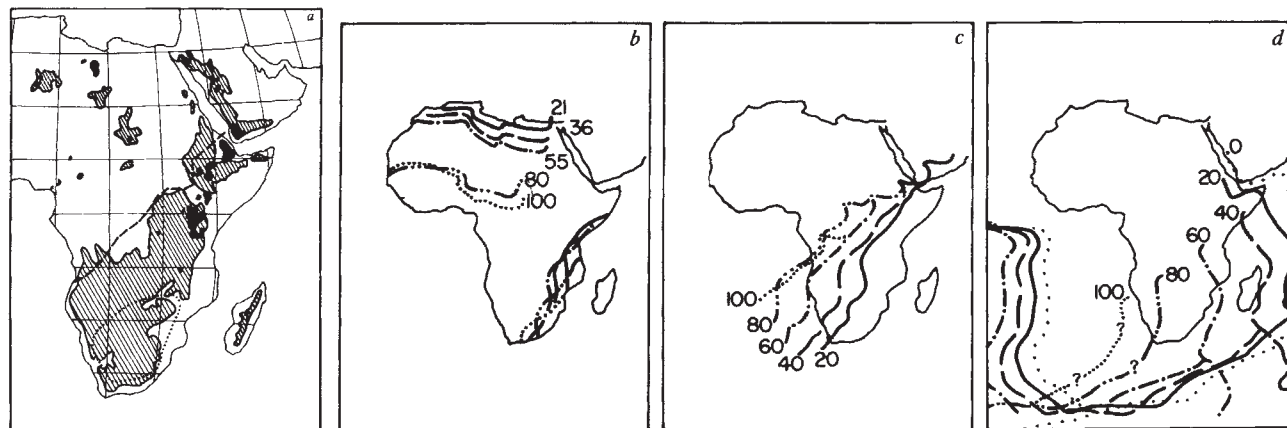


Fig. 1 *a*, Africa showing the distribution of Cenozoic volcanic rocks (black); the Karroo igneous rocks (within dotted line); land higher than 1,000 m (shaded); 80-Myr boundary of Africa relative to South America (dashed line). Note mutual exclusion of Karroo and Cenozoic volcanism; close similarity of northwestern edge of high Africa (essentially the 1,000-m contour) and 80 Myr boundary of Africa relative to South America. (Lambert equal area projection centred on 0° N, 15° E.) *b*, The past positions of Africa in the hotspot reference frame¹⁸. There is no correlation between these positions and the boundary between high and low Africa. In *b*, *c* and *d* numbers are ages in Myr and cylindrical equidistant projection is used. *c*, Successive former positions of eastern edge of Afro-Arabia relative to South America superimposed on present-day Africa. *d*, Successive former positions of north-west Indian Ocean ridge under present-day Africa in past 60 Myr. The ridge positions at 80 and 100 Myr are implied by continental reconstructions, but are not known from ocean-floor anomaly data. The ridges are shown relative to a fixed South America.

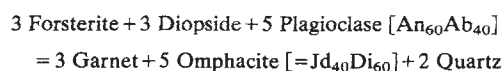
For example, if it is assumed that the asthenosphere under the rift valleys is essentially at the Moho⁷, then a 150-km lithosphere under low Africa implies that much of the upper mantle under the rift valleys has been reduced in density by 26 kg m^{-3} . (Brown and Girdler⁷ adopted a thinner reference lithosphere (100 km), which requires a density contrast of 40 kg m^{-3} .) Using representative values of specific heat and thermal expansion¹², it can be shown that the heat required to bring about this density decrease by expansion alone is about $1.6 \times 10^{11} \text{ kJ m}^{-2}$ of surface area, implying an average temperature increase of 450°C , which is absurdly high.

Similarly improbable values are obtained from heat flow calculations. For example, the temperature increase at the base of a 150-km thick lithosphere needed to cause 1,200 m of uplift is about $1,000^\circ \text{C}$ (ref. 5), which is far too high. Alternatively, 1,200 m of uplift will take at least 100 Myr (ref. 5) if the heat flux at the base of a 150-km plate is increased by $\sim 94 \text{ mW m}^{-1}$, about twice the average continental heat flow. All these thermal models require the crust under southern Africa to have partially melted, creating abundant magmatism in an area that is notably free from it. Despite their success in explaining ocean-floor subsidence², conduction uplift models cannot account for rapid plateau uplift of continents.

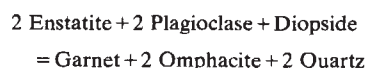
Phase change?

I suggest that the uplift of high Africa is most reasonably interpreted as a phase change. I assume the phase change to be analogous to the basalt/eclogite phase change and to occur at the Moho itself, taken initially at 40 km depth. The density change is from a relative density of 3.4 to 3.0. To create 1.2 km of uplift, 9 km of eclogite will have been converted to 10.2 km of 'basalt'. An analogous reaction is that of quartz eclogite to granulite.

Both reactions can be idealized, the first as (in moles)



and the second as:



The enthalpy change for the quartz eclogite to granulite reaction can be estimated from thermochemical data at 10^5 Pa (refs 13, 14) and extrapolated to high pressures. It probably lies between

-24.7 kJ kg^{-1} at 10^9 Pa and -46.0 kJ kg^{-1} at $1.5 \times 10^9 \text{ Pa}$. The enthalpy change for eclogite to 'basalt' at atmospheric pressures is very similar to that of quartz eclogite to granulite and is assumed to have a similar range at high pressures. These values probably have an uncertainty of 8 kJ kg^{-1} . For simplicity I adopt a value of 46 kJ kg^{-1} . The total heat required to convert 9 km of 'eclogite' to 10.2 km of basalt is $1.4 \times 10^{14} \text{ kJ m}^{-2}$ of cross-section. Thus the thermal energy needed for uplift due to a phase change is 100 times more effective than the energy needed for thermal uplift. The heat needed for a phase change uplift is equivalent to increasing the average temperature of the lithosphere by 4°C accompanied by 11 m of uplift. Such a small uplift cannot yet be detected in the residual depth anomalies of the adjacent ocean-floor. However, unlike thermal expansion, the active position of a phase change will be limited to a relatively narrow zone, rather than affecting all rocks, whatever their nature.

The numerous Mesozoic eclogite-bearing kimberlite pipes of southern Africa¹⁵ demonstrate the presence of eclogite in Mesozoic time in the upper mantle of southern Africa. Those eclogites containing diamonds come from much deeper levels, but others probably lay in the depth range of a possible phase change. The grain textures in some of the kimberlite inclusions show that during the Cretaceous there was a marked textural discontinuity at $\sim 150 \text{ km}$ depth where the temperature is estimated to have been $\sim 1,200^\circ \text{C}$ (ref. 10). This textural discontinuity implies a rapid stress drop at this level and can be taken as marking the Cretaceous lithosphere-asthenosphere boundary. Thus 70 Myr or more ago, eclogite was present in the upper mantle of southern Africa, and the lithosphere was

Table 2 Instantaneous absolute rotations of the American plate for three different models¹⁹

Model parameters	Lat.	Long.	Rotation rate ($10^{-7} \text{ deg yr}^{-1}$)
B4: continents have 3 times more drag than oceans	-62.0	-108.2	1.65
C4: drag under plates; slab pull at oceanic and continental subduction zones	-46.5	-89.5	1.72
D1: maximum pull by slabs and plate drag	-86.9	-61.3	0.89

150 km thick. How much eclogite was present and where it was distributed are not known, but similar kimberlite diatremes occur in the Colorado Plateau and in south-east Australia^{16,17}. In both these regions the mantle in the 40–70-km depth range might have contained up to one-third eclogite and other high pressure assemblages capable of inverting to lower density rocks^{16,17}.

The thermal time constant due to temperature changes of the continental lithosphere is ~63 Myr (ref. 9). For a phase change at the Moho, the time constant for heat originating at the base of the lithosphere is smaller. The minimum extra heat flux required for a phase change is the heat needed divided by the time available, or 4.6 mW m^{-2} . This heat is the minimum extra heat flux required above the value prevailing just before 10 Myr, because 10 Myr or so ago the latest phase of African uplift was just about to start. It would be less if uplift started earlier (C. Hartnady, personal communication). Clearly, a phase change overcomes the temperature problem, but it would not be expected to give such a rapid uplift pulse as is inferred from the geomorphology. Presumably, the sharpness of this uplift is caused by simultaneous thermal thinning of the lithosphere by the heat pulse as it moves upward⁵. Thus we are looking for a thermal pulse originating below Africa some time ~70 Myr ago, that probably thinned the lithosphere—because the isotherms would have been raised—and has augmented the previous heat flux at ~40 km by at least 4.6 mW m^{-2} , or about one-tenth of the average continental heat flow.

The increase in surface heat flow will be much less because the phase change itself will initially absorb much of the heat. The increase in temperature, as opposed to the minimum increase in heat flux, is difficult to estimate. However, a maximum temperature increase of 100 °C is suggested.

South America as a reference frame

To investigate this problem further we need a reference frame. The hotspot reference frame¹⁸ shows no relationship between possible hotspots or other hot areas and areas of uplift in southern Africa (Fig. 1b), presumably because hotspots do not cause plateau uplift. The hotspot reference frame is therefore rejected. Absolute reference frames are those in which there is no net rotation of the lithosphere¹⁹. Palaeomagnetic data suggest that for the past 50 Myr the equatorial components of any net finite rotation of the lithosphere relative to the mean global palaeomagnetic pole are perhaps as small as 2° (refs 20, 21). Because of the longitude indeterminacy, palaeomagnetic data cannot be used to find the polar component of any such rotation.

Nevertheless, I take here as reference frame that continent which has moved least relative to the global palaeomagnetic pole in the past 100 Myr, realizing that a finite rotation of arbitrary magnitude about the pole can be superimposed at any time on such a continent without being palaeomagnetically detectable. The continent that has moved least relative to the global palaeomagnetic pole is South America (Table 1)²². That

Table 3 Angular differences between instantaneous 'absolute' rotation poles and finite rotation poles for 55-Myr reconstruction

Fixed continent	Continent moved	Differences (deg) between finite rotation pole and instantaneous 'absolute' rotation pole		
		Model ¹⁹		
		B4	C4	D1
Antarctica	Africa	37.8	32.7	51.9
Eurasia		47.6	47.3	41.2
North America		36.6	41.9	18.6
South America	Australia	15.5	20.3	4.3
Antarctica		22.8	24.9	24.0
Eurasia		19.6	21.8	20.9
North America		6.5	11.1	4.3
South America		7.5	4.1	11.3

is, its average distance from the pole and the average distance moved during successive intervals are smaller than that for any other major continent.

Independent support for taking South America as a reasonable approximation to an absolute reference frame is provided by absolute motion studies¹⁹ (Table 2). Except for the Caribbean plate, in five out of eight models¹⁹ examined, the American plate (combining North and South America into one plate) has the smallest r.m.s. absolute velocity¹⁹. South America alone would have the smallest r.m.s. absolute velocity of all the larger plates because it generally lies close to the American rotation pole (Table 2). Inspection of the velocity field for model A3 of Solomon *et al.*'s¹⁹ also shows the very small linear velocities of South America.

South America was adopted as a reference frame because it approximates to the present absolute reference frame, and the rotation poles support this choice. Lithospheric thickening and subduction slab pull seem to account for most of the driving forces on a plate²³. In the case of the Australian and African plates the gross relative geometry of ridges and subduction zones has not changed much in the past 55 Myr. Before then, Australia was part of the Antarctic plate and a very different plate geometry existed^{21,24}. Therefore, we might expect that, to a first approximation, the instantaneous rotation poles in the absolute reference frame are not far from the finite poles for the period 0–55 Myr. We know the finite poles relative to the continental plates²². The plate that moves least relative to the absolute reference frame is likely to be that plate with respect to which the calculated finite rotation poles of Australia and Africa are closest to their present-day instantaneous absolute rotation poles.

The three absolute motion models¹⁹ closest to Harper's successful driving force model^{24,25} were chosen (B4, C4 and D1)¹⁹ and the results compared (Table 3). (D1 gives too high a net rotation of the lithosphere, but is retained for interest.) Clearly, the assumed finite pole of Australia in the absolute reference frame is equally close to the actual finite rotation pole in a reference frame fixed to either of the Americas. But only South America gives an actual finite rotation pole which is close to the assumed finite absolute reference frame pole for Africa.

During the period 100–0 Myr, Africa has rotated anticlockwise relative to South America (Fig. 1c, d). In particular, those areas affected by plateau uplift now overlie or have passed across the former positions of the oceanic ridges that separated Madagascar and India from Africa in the same period. There is, in fact, a remarkable coincidence between the area of high Africa and the area swept out by these oceanic ridges in a reference frame fixed to South America (Fig. 1a, c, d). The high-low Africa boundary essentially coincides with the trace of the southeastern edge of Africa at 80 or 60 Myr on present-day Africa (Fig. 1c, d). The hypothesis is that a thermal pulse, originating in the underlying asthenosphere at ~70 Myr, caused uplift about 50–60 Myr later. Imposing the small rotation needed to put South America into the present absolute reference frame (Table 2), assuming it has been unaltered for 70 Myr, would not alter this visual correlation between uplifted areas and former ridge positions.

Late Cenozoic uplift and volcanism of south-east Australia

I test this hypothesis by examining the uplift history of a second otherwise stable continent that also overrode former oceanic ridge positions in a South America reference frame: south-east Australia. Relative to South America, south-east Australia moved onto the former ridge position in the Tasman Sea shortly after it had opened between 76 and 56 Myr (refs 26, 27). If the Australian lithosphere is similar to Africa, I expect strong uplift beginning ~16 Myr ago.

The south-east Australian highlands form a 300-km wide tract rising to over 1,000 m above sea level, whose western boundary is roughly parallel to the trace of the western edge

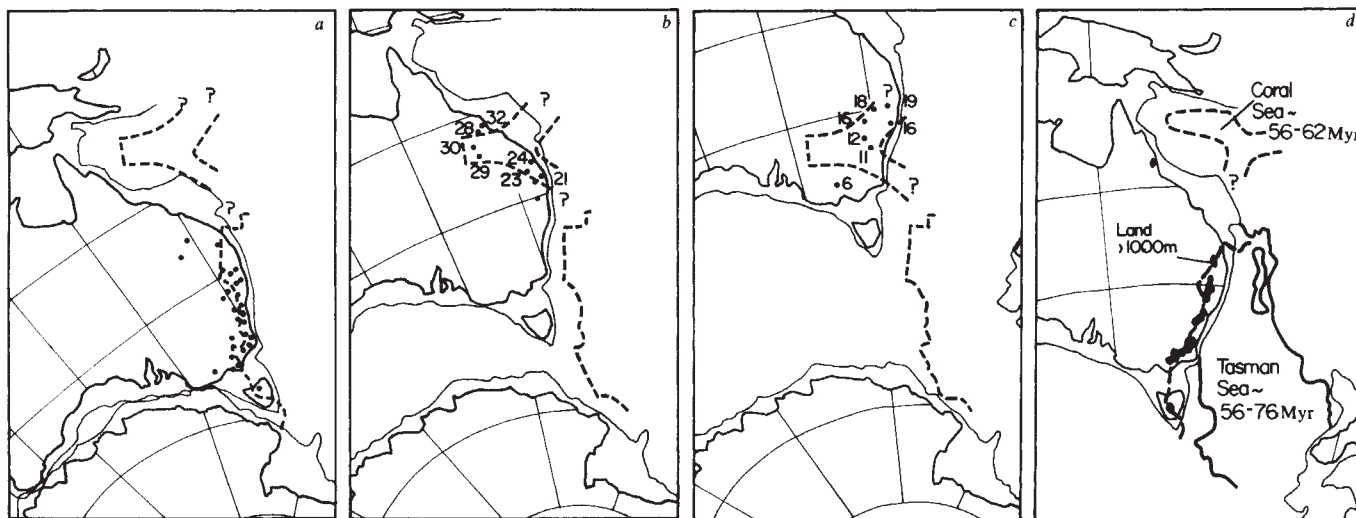


Fig. 2 *a*, Australia relative to South America at 55 Myr. The thick dashed lines show the approximate positions of the edges of Tasman Sea and the Coral Sea generated earlier relative to South America. ●, Central volcanoes, ages in Myr (ref. 34), whose activity reached a peak in 55–34 Myr. Note the coincidence between these volcanoes and the position of the Tasman Sea ridge in a reference frame fixed to South America, with a time-lag of perhaps 20–30 Myr. *b*, As *a* but at 40 Myr. Note how the inferred position of the Coral Sea ocean floor at 40 Myr in a reference frame fixed to South America coincides with lava field provinces formed some 13 Myr later. In *b* and *c*, numbers are ages of alkaline central volcanoes in Myr (ref. 34). *c*, As *a* but at 20 Myr. Note how the inferred position of the Coral Sea ridge positions fixed to South America at 20 Myr coincides with the lava field provinces formed some 6 Myr later. *d*, Present-day map showing Australia; the approximate age and distribution of ocean floor in the Coral Sea and Tasman Sea; the trace of the western edge of Tasman Sea from *a* and land over 1,000 m (black areas). Note the approximate coincidence of the highlands uplifted in Oligocene or later time, with the trace of the western edge of the Tasman Sea of early Tertiary age fixed to South America. *a*, *b*, *c* Are the same windows on Lambert equal-area maps in which South America is fixed; *d* is a window on a present-day Lambert equal-area map.

of the Tasman Sea at 76 Myr under Australia at 55 Myr, both in a South American fixed reference frame (Fig. 2*d*). Most studies conclude that at least in the south-east 80% or more of the uplift has taken place since the Oligocene (more recently than 22 Myr)^{28–31}, though some authors believe it to be much older³², or to have started earlier and to have been constant with time³³. Neighbouring areas have not been affected by so marked an uplift. Thus the area affected by uplift and the consensus of geomorphic interpretations fit well with the hypothesis.

Unlike high Africa, the south-east Australian highlands are closely linked to volcanism: a linear zone of basaltic lava fields is roughly coincident with the highland crest^{34,35} (Fig. 2*a*). Where data allow interpretation, a westward component is seen in the migration direction of this volcanism³⁴, which is expected if the heat sources are fixed in a South American reference frame.

At ~55 Myr, Australia started to move northwards relative to South America at ~53 mm yr⁻¹, eventually bringing the southeastern highlands region onto the former positions of the ridges in the Coral Sea, which was also relatively young³⁶ (Fig. 2*b*, *c*). A suite of alkalic central volcanoes formed, starting in the north at ~30 Myr, and migrating south to reach Victoria ~6 Myr ago, with an average migration rate of 66 ± 5 mm yr⁻¹ (ref. 34) (Fig. 2*c*). Like the basaltic lava fields, there is a similar time lag between the arrival of the affected parts of the highlands over the Coral Sea ridge positions and subsequent alkaline volcanism (Fig. 2*b*, *c*).

The similarity in timing between the lava field volcanism and the opening of the Tasman Sea has been noted previously^{34,35}, but no precise cause has been proposed. Likewise, the "remarkable migration of eruptive centres southwards (66 mm yr⁻¹) with time at a rate similar to the drift of Australia northward from Antarctica (56 mm yr⁻¹)" has been related to possible plumes and/or hotspots in the underlying asthenosphere^{34,35}. However, the northward migration of Australia relative to South America is at a similar rate, 53 mm yr⁻¹.

I propose that instead of asthenospheric plumes or hotspots causing magmatism, that the magma source originally lay within the Australian lithosphere. As a heat pulse moved upwards from the former positions of the Tasman and Coral Sea ridges,

the lithosphere thinned, eventually bringing the source region into the asthenosphere. As there seems to be a 20-Myr time lag between overriding of the continent and the onset of volcanism, I assume that the source originally lay well above the base of the lithosphere. Taking 65 Myr as the time constant for conductive heat transport for the whole continental lithosphere and a thickness of ~150 km, I estimate that a 20-Myr time lag probably implies a source depth of ~70–110 km below the surface.

The zone causing uplift some 60 Myr after Australia overrode the former positions of the western edge of the Tasman Sea must lie at much shallower levels, presumably in a zone capable of a phase change in the uppermost mantle. South-east Australian kimberlites, mostly of Jurassic age, include eclogites and other inclusions¹⁷. Refraction seismic lines show too that the present-day lithosphere in south-east Australia has a broad transition from crustal to mantle velocities, which could correspond to a zone of phase change³⁷.

Discussion

Provided that the former ridge positions also mark the locations of slightly hotter asthenosphere, the physical requirements of a phase change origin for African uplift lead to conditions that can be satisfied by the kinematics in a South American reference frame. I postulate that during the interval 80–40 Myr or so, what is now high Africa moved onto asthenosphere that had risen towards the surface as a direct result of Indian Ocean spreading. This asthenosphere was slightly hotter than the original asthenosphere under high Africa and created a heat pulse at the base of the African plate, which was too small to have a significant effect on the depth of the ocean floor, but was large enough to cause a phase change (or phase changes) in the uppermost 30 km or so of the continental mantle some 40–80 Myr later. Although three-dimensional mantle circulation models do not embody the complexities found in reality^{38–40}, cross-sections through such a circulation model in the present absolute reference frame at the present-day Carlsberg Ridge^{38,39} show the situation envisaged: deeper, and therefore hotter, asthenosphere rising up to replace preexisting asthenosphere under the edge of the African plate, which the plate will

eventually cover. In the absolute reference frame the ridges must move relative to their immediately underlying asthenospheric sources¹⁹.

What is not clear is why the past positions of the ridges in the approximate absolute reference frame provided by South America seem to match the areas of uplift so well. One possibility is that because asthenospheric sources are probably vertically extensive^{38,39} and have horizontal velocities much less than the overlying plates³⁸⁻⁴⁰, the asthenospheric zone extending from vertically above the deep source of a new ridge to the present position of the ridge may all have become hotter than it was before ridge creation. If so, this increased temperature could provide the heat needed for the phase change.

In detail, this model must be modified. For example, the uplift of southern Africa has been treated as a uniform vertical uplift simultaneously affecting all of high Africa, yet it is quite clear from Fig. 1c, d that we would expect a wave of uplift to have moved across high Africa starting in the east, and taking a geologically significant time to reach the west. This problem will not be discussed here in detail because part of its solution depends on considering still earlier phases of uplift probably resulting from the breakup of Gondwanaland. Nevertheless, it is worth noting that there is a distinct structural and topographic

asymmetry to southern Africa. The Cenozoic Kalahari basin lies in the western half of southern Africa, while the eastern half consists primarily of Precambrian rocks: during much of Cenozoic time, the eastern half of southern Africa has been structurally raised relative to the western half. In addition, the topography is markedly asymmetric: the highest parts of southern Africa are in Lesotho, very close to the eastern margin. Here, and nowhere else, the topography rises to well over 3 km. This structural and topographic asymmetry is what would be expected from a more detailed application of the model. The same mechanism is envisaged for the uplift of south-east Australia, where the expected topographic asymmetry is quite clear.

All these speculations can be tested by better data from the uplift of land surfaces, high-pressure petrology and three-dimensional mantle flow models.

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Isolation and preliminary characterization of a human transforming gene from T24 bladder carcinoma cells

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DNA from T24, a cell line derived from a human bladder carcinoma, can induce the morphological transformation of NIH 3T3 cells. Using techniques of gene rescue to clone the gene responsible for this transformation, we have found that it is human in origin, <5 kilobase pairs in size and is homologous to a 1,100-base polyadenylated RNA species found in T24 and HeLa cells. Blot analysis indicates extensive restriction endonuclease polymorphism near this gene in human DNAs.

THE progression of a cell lineage from normalcy to malignancy may involve the mutation or activation of one or more genes. The genomes of retroviruses contain candidates for such 'oncogenes'. Certain retroviruses capable of inducing neoplasia *in vivo* and cell transformation *in vitro* contain transduced cellular genes which entirely encode the oncogenic proteins of these viruses^{1,2}. If these or other oncogenes are expressed in tumours of viral or nonviral origin, the introduction of these

genes into cultured cells might transform the recipients and render them tumorigenic. Indeed, DNA from some chemically transformed mouse cells can morphologically transform NIH 3T3 mouse fibroblasts following DNA-mediated gene transfer³. More recently, it has been reported that DNA from certain human tumour cell lines can also morphologically transform NIH 3T3 cells^{4,5}. We have detected transforming activity in DNA from 5 of 21 human tumour cell lines⁶; the resulting

NIH 3T3 transformed cells are tumorigenic in nude mice⁴⁻⁶. Human DNA from one colon and two lung carcinoma cell lines appear to contain the same or highly related transforming elements, while DNA from a bladder carcinoma and from a neuroblastoma cell line each contain different transforming elements⁶. These transforming genes may have played a critical part in the origins of the tumours from which these cell lines derive. Here, we report the molecular cloning and preliminary characterization of the transforming gene from the human bladder carcinoma cell line, T24 (ref. 7).

Strategy of gene isolation

In principle, a gene can be isolated whenever its transfer into an appropriate host results in the stable acquisition of a selectable phenotype, using either 'search' or 'rescue' strategies. In the former, a complete genomic library is constructed in plasmids, phage or other cloning vehicles, and individual members of the library are screened by DNA-mediated gene transfer. This approach has been used successfully to isolate genes from lower organisms⁸. The rescue strategy entails cleaving DNA from cells containing the gene with a restriction endonuclease and then ligating this DNA to a defined 'marker' sequence. The ligated DNA is then used in gene transfer experiments, ultimately yielding a cell bearing the transferred gene linked to the marker sequence. The gene is isolated by one of several means on the basis of its association with the marker. The chicken thymidine kinase (tk) and hamster adenine phos-

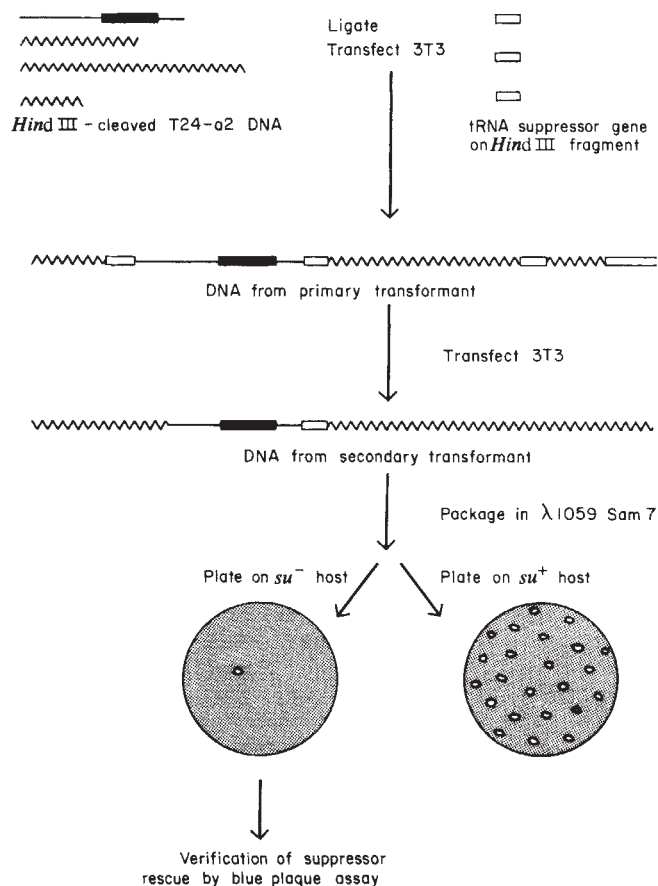


Table 1 Transfection of NIH 3T3 cells with uncleaved and restriction endonuclease-cleaved DNAs from T24 and T24-a2 cell lines and recombinant bacteriophage and plasmid

a	Suppressor rescuant	Foci per 0.25 µg DNA
	λsup2.9	86
	λsup1.12	0
	λsup5.3	0
	λsup5.4	0
b	λsup2.9 (Foci per 0.25 µg)	pTB-1 (Foci per 0.05 µg)
Undigested	86 (1.5×10^{-8})*	372 (1×10^{-7})*
BamHI	35	NT
BglII	71	NT
EcoRI	176	NT
HindIII	91	NT
XbaI	0	NT
	T24 (Foci per 70 µg)	T24-a2 (Foci per 20 µg)
Undigested	11 (5×10^{-7})*	6 (9×10^{-7})*
BamHI	15	8
BglII	7	4
EcoRI	7	34
HindIII	13	10
XbaI	0	0

DNA (total concentration $30 \mu\text{g ml}^{-1}$) was precipitated with calcium phosphate and 1 ml of suspension was applied to 10^6 NIH 3T3 cells in a 10-cm culture dish, as previously described¹². Restriction endonuclease-digested T24 and T24-a2 DNAs were mixed in a 1:2 ratio with uncleaved NIH 3T3 DNA before precipitation; samples of phage and plasmid DNAs were added to $30 \mu\text{g}$ NIH 3T3 DNA before precipitation. Cellular DNAs were prepared by SDS-proteinase K lysis and phenol/chloroform extraction⁶. Phage DNAs were prepared from CsCl gradient-purified virions¹⁸ and plasmid DNA was purified from saturated culture of bacteria¹⁹. a Shows the NIH 3T3 focus-forming activity of DNAs from four independent suppressor-containing phages; b compares the focus-forming activity of λsup2.9 DNA and pBR322 subclone pTB-1 DNA, and compares the restriction endonuclease sensitivity profile of the activity in λsup2.9 DNA with the previously reported⁶ activities in T24 and T24-a2 cellular DNAs. NT, not tested.

* Value in parentheses is the calculated frequency of focus induction per transforming gene equivalent. In these calculations, we have taken the molecular weight of the human haploid genome to be 1.5×10^{12} .

Fig. 1 Suppressor rescue of the T24 transforming activity. A recombinant DNA plasmid, pK-7, containing a *supF* tRNA amber suppressor gene was given by Dr U. RajBhandary. The suppressor gene was further subcloned into pBR322 plasmid (K.S. *et al.*, in preparation). This subclone, pK-5, was cleaved with restriction endonuclease *Hind*III, and the 1.1-kbp suppressor DNA fragment was purified by gel electrophoresis and binding to glass powder²⁰. T24-a2 is a transformed NIH 3T3 cell line derived from transfection of NIH 3T3 cells with T24 DNA. *Hind*III-cleaved T24-a2 DNA was ligated in fourfold mass excess to suppressor fragment DNA with T4 DNA ligase (New England Biolabs) at a total DNA concentration of $250 \mu\text{g ml}^{-1}$. Ligated DNA was mixed with a twofold mass excess of NIH 3T3 DNA and transfected to NIH 3T3 cells by methods described elsewhere¹². This ligated DNA induced foci at a threefold lower efficiency than native T24-a2 DNA in the absence of suppressor. Resulting transformed cells were cultured from individually picked foci. DNA from these cells should contain some *supF* sequences closely linked to the transforming element, but most *supF* genes, introduced by co-transfer, will not be closely linked. These DNAs were transfected again to NIH 3T3 cells, yielding secondary foci bearing only closely linked *supF* sequences. DNA from six secondary foci were partially digested with restriction endonuclease *Sau*3A, and 10–20-kbp fragments were purified by centrifuging $150 \mu\text{g}$ DNA through a 15–40% (w/v) sucrose gradient (in 100 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA) at 23,000 r.p.m. in a Beckman SW27 rotor for 24 h at 20 °C. From the six individual size-selected DNA preparations, a total of $5.3 \mu\text{g}$ DNA fragments ($250 \mu\text{g ml}^{-1}$) were ligated to a twofold mass excess of gel-purified phage DNA arms generated by cleavage of λ1059Sam7 DNA with endonuclease *Bam*HI. λ1059Sam7 was derived from *in vivo* recombination between λ1059 and λSam7 (K.S. *et al.*, in preparation). Chimaeric phage DNA molecules were packaged into virions by a standard procedure¹⁵. A total of 8.4×10^6 phage particles were generated by packaging, as measured by titration on *E. coli* BNN45 *supE**supF* cells. These phages were plated onto *E. coli* KS624 *sup*⁰(*r*⁺, *m*⁺) cells (K.S. *et al.*, in preparation). Thirty-four individual plaques which arose were picked and the phages screened for the presence of amber suppressor by spotting on to lawns of *lacZ*^{am} *sup*⁰ cells in the presence of *lacZ* inducer isopropyl thiogalactoside and indicator substrate, Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; ref. 21). Suppressor-bearing phage suppressed the *lacZ*^{am} mutation, generating a blue plaque by β-galactosidase cleavage of Xgal. By this procedure, we isolated four suppressor-bearing phages. In the upper part of the figure, — denotes human DNA sequences; ~, mouse DNA sequences; ■, T24 transforming gene; □, tRNA *supF* gene.

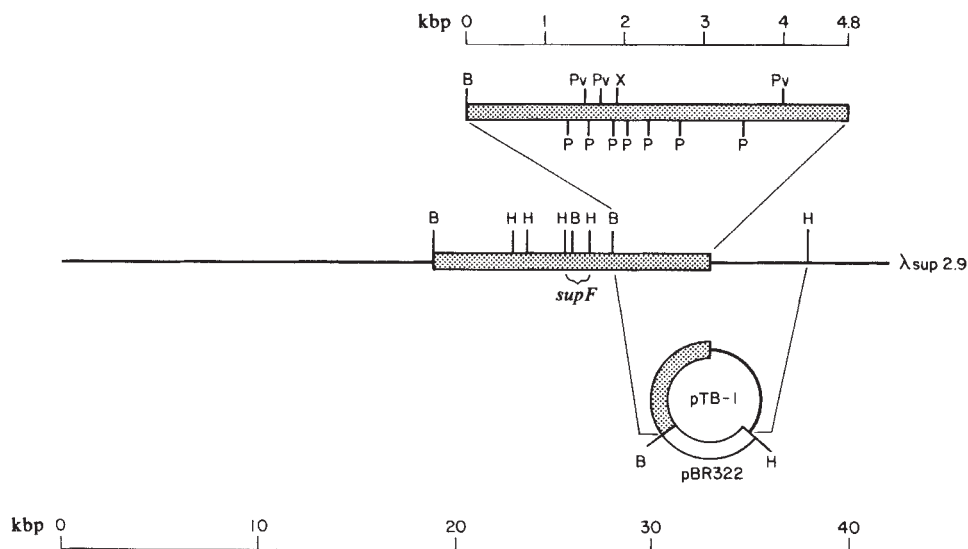


Fig. 2 Restriction endonuclease cleavage map of λ sup2.9 and pTB-1. The figure shows the restriction map of DNA from the recombinant bacteriophage λ sup2.9 which was active in transformation assays. Phage arms are denoted by a thin line, and the 14-kbp DNA insert by a shaded box. The suppressor DNA fragment is marked *supF*. Below is shown the circular map of pTB-1, with the *Bam*HI/*Hind*III fragment of λ sup2.9 cloned into pBR322, denoted by an open box. Above is shown a more detailed restricted map of the cloned T24 bladder carcinoma transforming sequences. The cluster of *Pst*I sites were mapped by the method of Smith and Birnstein²². There are no *Eco*RI, *Hind*III or *Bgl*II sites in this sequence. B, *Bam*HI; H, *Hind*III; P, *Pst*I; Pv, *Pvu*II.

phoribosyl transferase genes were isolated using the *Escherichia coli* plasmid pBR322 as the marker sequence^{9,10}.

In this study, we have adopted a modified rescue strategy using an *E. coli* tRNA amber suppressor gene, *supF*, as marker. Details of this method will be published elsewhere (K.S. *et al.*, in preparation) but the scheme is outlined in Fig. 1. From pK-7, a plasmid which contains *supF*, we constructed a new plasmid from which the *supF* gene could be recovered as a 1.1-kilobase pair (kbp) DNA fragment following cleavage with *Hind*III. This suppressor DNA fragment was ligated in equimolar ratio to *Hind*III-cleaved DNA from T24-a2, an NIH 3T3 transformant containing the T24 transforming gene. *Hind*III cleavage does not affect the transforming activity of either T24 or T24-a2 DNA (see Table 1). We used T24-a2 DNA as our gene source because it has a higher specific activity of transformation than T24 DNA. Ligated DNA was mixed with a twofold mass excess of NIH 3T3 cellular DNA and added as a calcium phosphate precipitate to NIH 3T3 cells^{11,12}. Morphologically transformed foci were picked 2 weeks later and grown into mass culture. Blot hybridization analysis indicated that cells from these foci contained multiple copies of the bacterial tRNA gene. Presumably, at least one of these copies directly flanked the transforming gene while the others did not. Therefore, DNA from these transformants was again used for gene transfer to generate new foci. DNA from a few of the resulting foci retained one or two copies of the tRNA gene, and these DNAs were used in subsequent experiments.

To clone the tRNA suppressor gene and its flanking sequences, we used a biological selection based on the ability of the cloned tRNA gene to suppress amber mutations when incorporated into a mutant of bacteriophage λ . We introduced by molecular recombination an amber mutation (Sam7)¹³ into the lysis gene of λ 1059, a cloning vehicle which accepts 7–18-kbp DNA fragments having GATC3'OH cohesive ends¹⁴. The resulting phage, λ 1059Sam7, required a *supF* host to complete a lytic cycle. DNA from transformed animal cells containing one to two copies of *supF* were partially digested with restriction endonuclease *Sau*3A and ligated to purified arms of λ 1059Sam7 DNA generated by *Bam*HI cleavage. The ligated DNAs were packaged into phage particles¹⁵ and plated onto *sup*⁰ and *supF* hosts. Phages which grew on *sup*⁰ hosts were further identified as *supF*-containing by their ability to convert a *lacZ*^{am} *sup*⁰ host to *lacZ*⁺. From such a screening process, we obtained four independent *supF* phage recombinants.

Cloned sequences transform NIH 3T3 cells

DNA from the four *supF* phages were mixed with NIH 3T3 carrier DNA and assayed for the ability to transform NIH 3T3 cells. As shown in Table 1, λ sup2.9 DNA induced transformed

foci at an efficiency of ~300 foci per μ g. The three other phage DNAs were inactive in transformation. As a first step in verifying whether the λ sup2.9 transforming activity is the same as that in T24 DNA, the activity of λ sup2.9 cleaved with restriction endonucleases was compared with similarly cleaved T24 DNA. Table 1 shows that the transforming activity of λ sup2.9 DNA is destroyed by cleavage with *Xba*I but not with *Bam*HI, *Bgl*II, *Eco*RI or *Hind*III. The same is true for T24 DNA.

To localize the transforming sequences of λ sup2.9, we subcloned restriction fragments of λ sup2.9 into pBR322. Cleavage

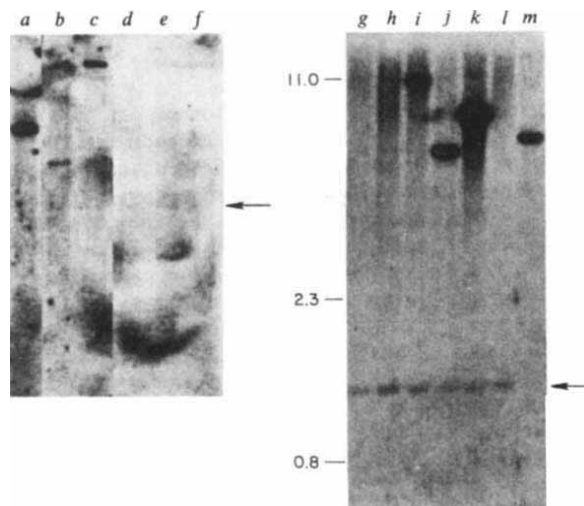


Fig. 3 Hybridization of pTB-1 DNA and one of its restriction endonuclease fragments to filter-blotted DNA from transformed NIH 3T3 cells. DNAs from NIH 3T3 cells, transformed NIH 3T3 cells⁶ and T24 cells were cleaved with either *Hind*III or *Bam*HI restriction endonuclease. Digested DNAs (6 μ g of each) were electrophoresed through 1.0% agarose gels and transferred to nitrocellulose filters as previously described¹⁶. Blotted DNAs were hybridized with nick-translated ³²P-DNA from either pTB-1 or the 800-bp *Pst*I fragment of pTB-1. Hybridizations were performed at 74 °C for 16 h in 6 × SSC, 1 × Denhardt's solution and 20 μ g ml⁻¹ denatured salmon sperm DNA²³. *Hind*III-cleaved cellular DNAs probed with pTB-1 ³²P-DNA (20 ng ml⁻¹) (a–f); *Bam*HI-cleaved DNAs probed with the *Pst*I fragment ³²P-DNA (2 ng ml⁻¹) (g–m). d, l, DNA from NIH 3T3. Secondary transformants of NIH 3T3 transformed with T24 DNA: T24-a1-3 (c, k); T24-a2-1 (a, j); T24-a5-4 (b, i). e, h, Secondary transformant of NIH 3T3 transformed with DNA from Calu-1, a human lung carcinoma cell line. f, g, Primary transformant of NIH 3T3 transformed with DNA from SK-N-SH, a human neuroblastoma cell line. m, T24. Hybridization of probe to *Hind*III-cleaved λ sup2.9 DNA and *Pvu*II- or *Pst*I-cleaved pTB-1 DNA provided molecular weight standards as indicated (in kilobase pairs).

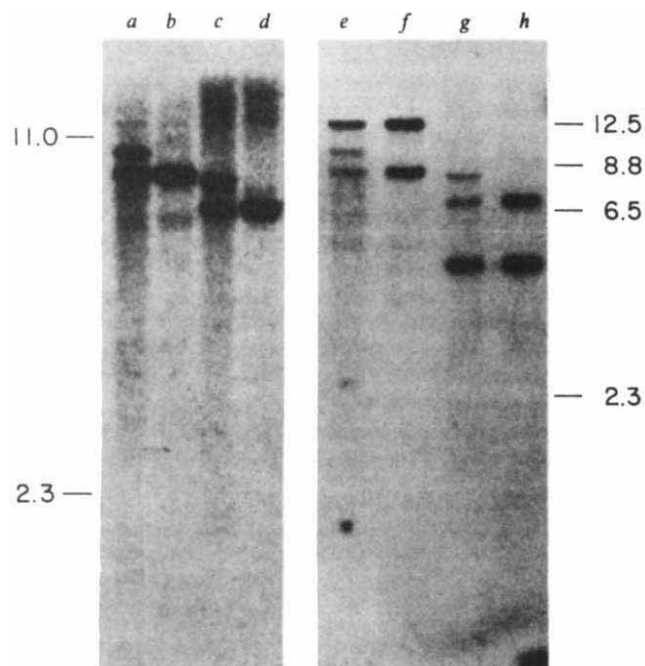


Fig. 4 Hybridization of pTB-1 DNA and its 800-bp *Pst*I fragment to filter-blotted DNA from T24 cells and human placenta. Human placental DNA (given by J. Fiddes) and T24 DNA were cleaved with restriction endonucleases, electrophoresed through 1.0% agarose gels and transferred to nitrocellulose filters. Blotted DNAs were hybridized with 20 ng ml⁻¹ pTB-1 ³²P-DNA (e-h) or 2 ng ml⁻¹ gel-purified, ³²P-labelled 800-bp *Pst*I pTB-1 DNA fragment (a-d). *Bgl*II-cleaved placenta DNA (a, e); *Bgl*II T24 (b, f); *Bam*HI placenta (c, g); *Bam*HI T24 (d, h). Hybridization of probes to *Xba*I- or *Hind*III-cleaved λ sup2.9 DNA and *Pvu*II-cleaved pTB-1 DNA provided molecular weight standards (in kilobase pairs).

of λ sup2.9 DNA with *Bam*HI and *Hind*III generates seven restriction fragments, five of which contain heterogeneous ends (Fig. 2). These were then inserted between the *Bam*HI and *Hind*III sites of pBR322 DNA. When individual plasmid subclones were tested for their ability to transform NIH 3T3 cells, only subclone pTB-1 had transforming activity. pTB-1 DNA contains a 9.8-kbp inserted sequence spanning the rightmost *Bam*HI site in λ sup2.9 to the *Hind*III site within the right arm of the phage DNA. As this region contains 5.0 kbp of λ DNA sequences, the transforming activity in pTB-1 is encoded in a transforming sequence no larger than 4.8 kbp. A more detailed restriction endonuclease map of this 4.8-kbp region is shown at the top of Fig. 2. The specific transforming activity of pTB-1 was higher than that of λ sup2.9, but was consistently lower in a series of experiments than the specific activity calculated for the chromosomal gene in T24, assuming the gene is present in T24 as a single copy (for example, see Table 1). This agrees with our previous results for clones of the chicken tk gene which transfer at reduced efficiencies compared with the chromosomal gene⁹.

pTB-1 contains transforming sequences of T24 bladder carcinoma cells

Hybridization studies provide further evidence that we have cloned transforming sequences from the T24 bladder carcinoma cell line. DNAs from NIH 3T3 cells, NIH 3T3 cells transformed with T24 DNA and NIH 3T3 cells transformed with DNA from other human tumour cell lines⁶ were digested with *Hind*III, electrophoresed through agarose gels, and transferred to nitrocellulose filters by the procedure of Southern¹⁶. These studies used DNAs from secondary NIH 3T3 transformants resulting from two cycles of transfection of tumour cell DNA through NIH 3T3 cells. The filters were hybridized with nick-translated ³²P-labelled pTB-1 DNA. Figure 3 shows that pTB-1

sequences hybridize weakly to a specific *Hind*III fragment in DNA from NIH 3T3 cells and all its transformants (indicated by arrow at right of lane f). pTB-1 also hybridizes weakly to specific restriction fragments of normal rat cellular DNA (data not shown). In addition to these interspecies homologies, pTB-1 hybridizes strongly to a single *Hind*III fragment in each of three independent secondary transformants of T24 (Fig. 3, lanes a-c), while NIH 3T3 DNA shows no additional hybridization (lane d). We conclude that pTB-1 contains the transforming sequences present in T24 DNA. DNA from an NIH 3T3 secondary transformant derived from human lung carcinoma, Calu-1 DNA (Fig. 3, lane e) and DNA from an NIH 3T3 primary transformant derived from human neuroblastoma, SK-N-SH DNA (lane f), also lack additional sequences homologous to pTB-1, demonstrating that the transforming element in T24 differs from that in Calu-1 and SK-N-SH cells. This conclusion is consistent with our previous findings⁶.

Human placental DNA contains sequences homologous to the T24 transforming gene

If the transforming activity in T24 DNA arose by the acquisition of exogenous (for example, viral) genetic information, then

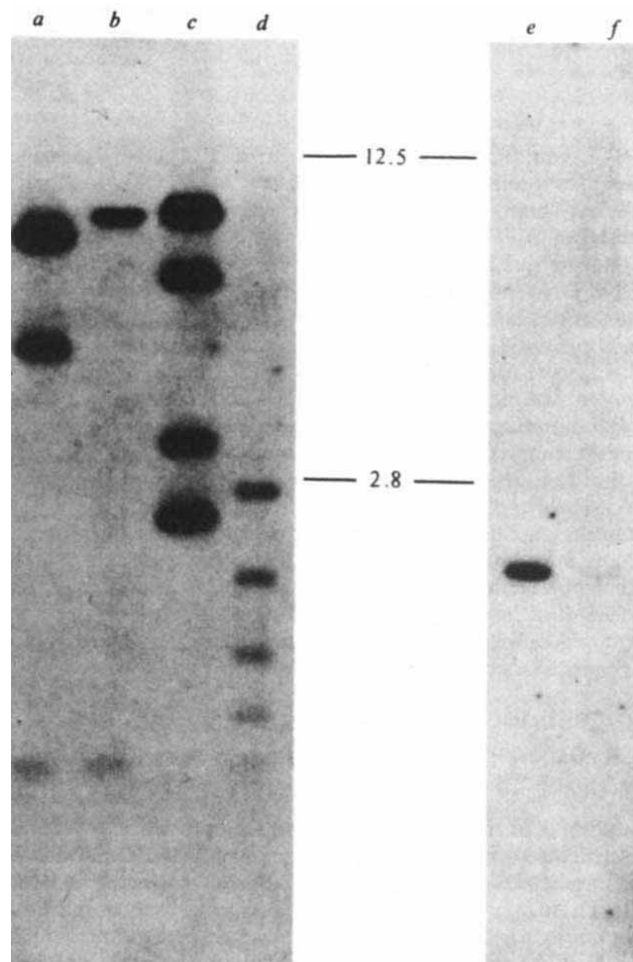


Fig. 5 Hybridization of pTB-1 DNA to filter-blotted T24 and pTB-1 DNAs cleaved with restriction endonucleases. T24 cellular DNA (6 μ g) and pTB-1 (50 μ g) were cleaved with restriction endonucleases, electrophoresed through a 1.0% agarose gel, then transferred to a nitrocellulose filter. pTB-1 DNA was cleaved with *Pst*I (a) and *Pvu*II (c); T24 DNA was cleaved with *Pst*I (b) and *Pvu*II (d). These blotted DNAs were hybridized to total pTB-1 ³²P-DNA (20 ng ml⁻¹). 20 μ g of the 1.9-kb purified fragment of *Bam*HI/*Xba*I-cleaved pTB-1 (e) and T24 DNA cleaved with *Bam*HI/*Xba*I (f) were hybridized to the ³²P-labelled 1.9-kbp *Bam*HI/*Xba*I pTB-1 fragment (5 ng ml⁻¹). Hybridization of probe to *Xba*I-cleaved λ sup2.9 DNA provided molecular weight standards (in kilobase pairs).

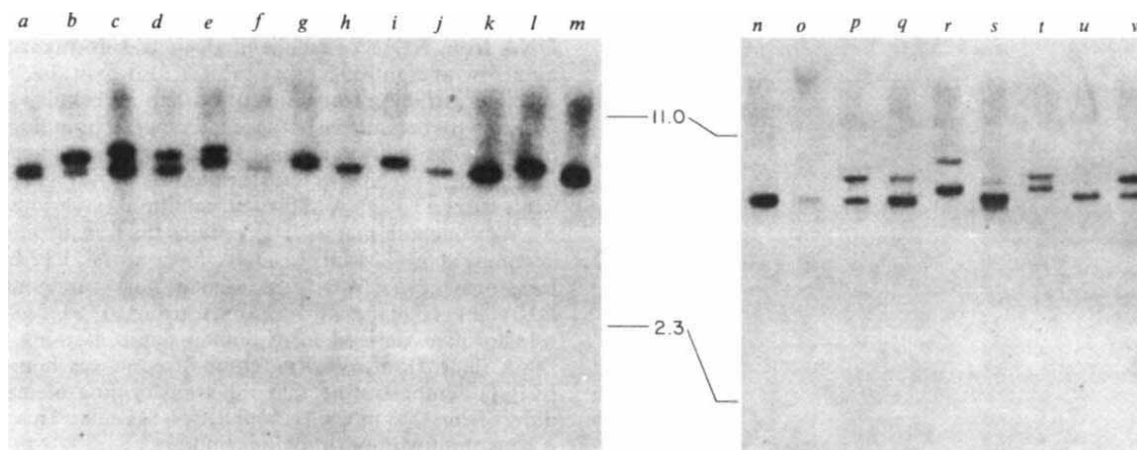


Fig. 6 Hybridization of the 800-bp *Pst*I fragment of pTB-1 DNA to filter-blotted DNAs from human tumours and tumour cell lines. Human tumour cell lines were given by J. Fogh. Human tumours were obtained from the NCI Tumor Repository. DNAs from human tumours and tumour cell lines were cleaved with *Bam*HI restriction endonuclease, electrophoresed through 1% agarose gels and then transferred to nitrocellulose filters. Blotted DNAs were hybridized to 2 ng ml⁻¹ gel-purified ³²P-labelled 800-bp *Pst*I pTB-1 DNA fragment. DNAs from tumour cell lines: a, epidermis RPMI 2650; b, colon HT-29; c, cervix C4II; d, glial cell T98; e, osteosarcoma 8387; f, breast MCF-7; g, cervix HeLa; h, bladder T24; i, melanoma RPMI 4445; j, fibrosarcoma SW594; k, bladder 575A; l, bladder 496P; m, bladder TCCsup; n, pancreas Capan-1; and o, bladder VM-CUB-2. DNAs from human tumours: n, colon 031-10687; o, lung 031-9337; p, lung 031-11971; q, colon 76-640; r, lung 031-13137; s, colon 021-769; and t, lung 76-126. Hybridization of the probe to *Hind*III-cleaved λ sup2.9 DNA and *Pvu*II-cleaved pTB-1 DNA provided molecular weight standards (indicated in kilobase pairs).

pTB-1 would probably hybridize to a T24 DNA sequence absent from human placenta DNA. To examine this possibility, T24 and human placenta DNAs were cleaved with either *Bam*HI or *Bgl*II restriction endonuclease, and digested DNAs were analysed by filter blot hybridization, using ³²P-labelled pTB-1 DNA as probe. Figure 4 shows that pTB-1 hybridizes to 8- and 12-kbp *Bgl*II fragments (lane f) and to 5.0- and 6.8-kbp *Bam*HI fragments (lane h) from T24 DNA. The probe hybridizes to *Bgl*II (lane e) and *Bam*HI fragments (lane g) of identical size in human placental DNA. We also observed that T24 and human placental DNAs have similarly sized *Eco*RI or *Hind*III fragments homologous to pTB-1 (data not shown). As all the T24 DNA restriction fragments homologous to pTB-1 have similarly sized counterparts in placental DNA, we conclude that the T24 transforming gene originated from human genetic information without the gross rearrangements of 'normal' DNA sequences. The fact that additional 9.5-kbp *Bgl*II and 7.9-kbp *Bam*HI fragments homologous to pTB-1 are present in placental DNA but absent from T24 DNA, we attribute to genetic polymorphism (see below).

Subgenomic fragments of pTB-1 suitable as hybridization probes for the T24 transforming gene

We wished to use pTB-1 as a hybridization probe to analyse the structure of the transforming gene in T24 and other human cell lines, and to monitor transcription of the gene in these cells. These applications require the human sequences in pTB-1 to be contiguous and not rearranged with respect to the chromosomal gene. Such rearrangements frequently occur after DNA-mediated transfer into animal cells¹⁷. We therefore compared the restriction endonuclease map of the human sequences in pTB-1 with that of the homologous sequences in T24 DNA. Filter hybridization of T24 DNA cleaved with *Bam*HI and *Xba*I to the ³²P-labelled 1.9-kbp pTB-1 *Bam*HI/*Xba*I fragment revealed a single homologous DNA fragment (Fig. 5f) co-migrating with the 1.9-kbp pTB-1 fragment (lane e), proving that the region extending from the *Bam*HI site to the *Xba*I site within the transforming gene is not rearranged in pTB-1. The 800-base pair (bp) *Pst*I fragment of pTB-1 (Fig. 5a) mapping between 2.7 and 3.5 kbp from the *Bam*HI site (see Fig. 2) can also be found in *Pst*I-cleaved T24 DNA (Fig. 5b), and this *Pst*I

fragment hybridizes to DNA restriction fragments unique to NIH 3T3 secondary transformants of T24 (Fig. 3i-k). These results demonstrate that the 800-bp *Pst*I fragment of pTB-1 is an unaltered component of the transforming gene or its flanking DNA sequences. However, the 2.3-kbp *Pvu*II fragment of pTB-1 (Fig. 5c) spanning 1.7 to 4.0 kbp from the *Bam*HI site is not present in *Pvu*II-cleaved T24 DNA (Fig. 5d), indicating that human DNA sequences more than 3.5 kbp from the *Bam*HI site in pTB-1 are rearranged. Thus, the 1.9-kbp *Bam*HI/*Xba*I and 0.8-kbp *Pst*I pTB-1 fragments were used as hybridization probes to study the structure and expression of the transforming gene.

Human DNAs are highly polymorphic near the T24 transforming gene

The restriction fragments of T24 and human placental DNAs homologous to the transforming gene were again compared, using the 800-bp *Pst*I fragment of pTB-1 as hybridization probe. The *Pst*I fragment hybridized only to the 8-kbp *Bgl*II fragment (Fig. 4b) and the 6.8-kbp *Bam*HI fragment (lane d) from T24 DNA. The probe detected identically sized *Bgl*II and *Bam*HI fragments from human placenta DNA (Fig. 4a,c), although the cleaved placenta DNA additionally contains a larger homologous fragment in each case. The presence of these larger fragments indicates the existence of genetic polymorphism near the transforming gene.

To measure the extent of this polymorphism, DNAs from 40 human tumours and tumour cell lines were cleaved with restriction endonuclease *Bam*HI and then analysed by gel electrophoresis and Southern blot hybridization, using the 800-bp *Pst*I fragment of pTB-1 as probe. The data for 21 of these DNAs are shown in Fig. 6. Sequences homologous to the probe were found in DNAs from all human sources, but the number and sizes of the homologous restriction fragments differed among the DNA samples. *Bam*HI-cleaved DNA from each human source contained one, two, or in one case, three discretely sized fragments ranging in molecular weight (MW) from 6 to 9 kbp. Among these DNAs, at least six different homologous *Bam*HI fragments were evident. We have also detected the same extent of restriction endonuclease polymorphism among normal placenta DNAs from different individuals (data not shown).

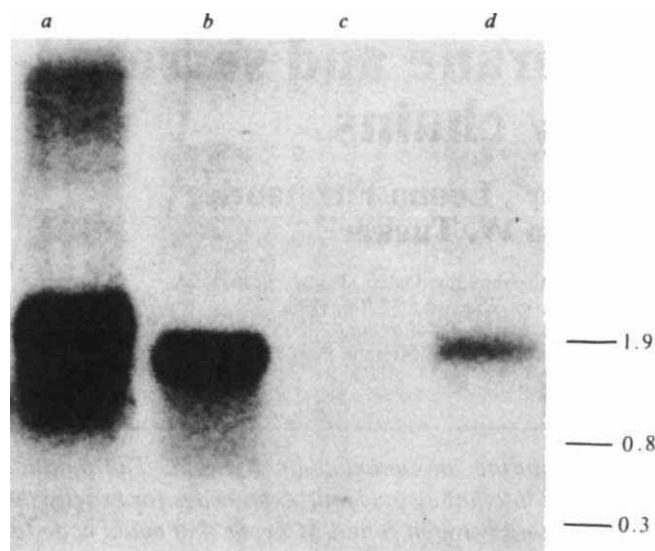


Fig. 7 Hybridization of restriction fragments of pTB-1 DNA to filter-blotted RNA. Total cellular RNAs were prepared by lysis of cells in 4 M guanidine thiocyanate²⁴ and removal of DNA and protein by extraction with hot phenol²⁵. Polyadenylated RNA was selected by oligo(dT)-cellulose chromatography; 5 µg of each poly(A)⁺ RNA were denatured in formaldehyde/formamide and electrophoresed through a 1.2% agarose gel containing 20 mM MOPS pH 7.0, 5 mM Na acetate, 1 mM EDTA, 2.2 M formaldehyde, and the RNA was transferred to a nitrocellulose filter, with 20× SSC as transfer buffer, as described by Thomas²⁶. The filter was hybridized at 42 °C in 5× SSC, 50% formamide, 10% dextran sulphate with 2 ng ml⁻¹ 800-bp *Pst*I pTB-1 ³²P-DNA fragment and 3 ng ml⁻¹ 1.9-kb *Bam*HI/*Xba*I pTB-1 ³²P-DNA fragment. Poly(A)⁺ RNA from 3T3 secondary transformant T24-a2-1 (a), T24 (b), NIH 3T3 (c), HeLa (d). *Bam*HI/*Xba*I-cleaved and *Pst*I-cleaved pTB-1 DNA provide molecular weight standards (in kilobases).

The hybridization intensities of the homologous *Bam*HI DNA fragments varied among the cleaved DNAs from different tumour sources. This variation is readily explained neither by inaccuracies of DNA concentration measurements nor by incomplete endonuclease digestions among the DNA samples analysed. We therefore suspect that the copy number of DNA sequences homologous to the T24 transforming gene varies about fivefold among the tumours and tumour cell lines.

Expression of pTB-1 sequences in transformed cells

To determine whether the transforming sequences are expressed in T24 cells and in NIH 3T3 cells transformed with T24 DNA, poly(A)⁺ total cellular RNA was prepared from T24 cells, NIH 3T3 cells, a T24 DNA-induced NIH 3T3 transformant and HeLa cells. The RNAs were electrophoresed in

formaldehyde/agarose gels, transferred to nitrocellulose filters and hybridized with a ³²P-labelled mixture of the 1.9-kbp *Bam*HI/*Xba*I fragment and the 0.8-kbp *Pst*I fragment of pTB-1 DNA. The results are given in Fig. 7. NIH 3T3 cells transformed by T24 DNA contain heterogeneously sized RNA species (1,500–1,800 bases) homologous to the pTB-1 fragments (Fig. 7a), while untransformed NIH 3T3 cells show no homology (lane c). A homologous RNA of discrete size (1,100–1,200 bases) is found in T24 cells (lane b).

The altered size of the transcripts found in NIH 3T3 transformants suggests that certain RNA processing signals in the transforming sequences were lost during gene transfer or are not recognized in the mouse cell host. A homologous RNA of the same size as that in T24 RNA is also found in the HeLa cells (Fig. 7d) but at reduced concentration. HeLa DNA does not transform NIH 3T3 in transfection assays. No homologous RNA species has been found in human placenta (data not shown). These results indicate that the transforming sequences are transcribed, that expression of these sequences is not restricted to T24 cells and that there is differential expression in cells from different sources. We estimate that transcripts of the T24 bladder carcinoma gene comprise 0.01% of poly(A)⁺ RNA from T24 cells.

Discussion

Previous studies using DNA-mediated gene transfer showed that DNA from T24 bladder carcinoma cells can efficiently induce foci of morphologically transformed cells in NIH 3T3 recipients⁶. In this study we have cloned from T24 DNA biologically active sequences which are responsible for transformation. Hybridization studies indicate that the transforming sequences contained in T24 are of human origin and closely homologous sequences are present in DNA from each of 40 different human sources that we have examined.

Of the 21 human DNAs screened, only 5 can efficiently transform NIH 3T3 cells⁶. T24 DNA transfers a different transforming element from the other four active donors; we conclude that the transforming sequences of T24 differ in some critical respect from the homologous sequences found in DNAs from other human sources. The transforming gene of T24 probably did not result from the gross rearrangement of normal sequences, as cleavage of T24 and human placental DNAs with *Bgl*II, *Bam*HI, *Hind*III or *Eco*RI yields similarly sized fragments that are homologous to the transforming gene. However, the extent of restriction endonuclease polymorphism at this locus in human DNA limits the strength of this conclusion. Further studies should elucidate the origins of the T24 transforming gene and determine whether its biological properties derive from the expression of an altered protein product or from an altered pattern of gene expression.

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Structure of genes for membrane and secreted murine IgD heavy chains

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We have sequenced secreted and membrane terminal exons of the murine immunoglobulin δ chain. The putative transmembranal exon is strikingly similar to that of the μ chain and the internal cytoplasmic exon codes for exactly the same two amino acids, valine and lysine. A third potential exon was found between S and M exons that could code for yet another hydrophilic carboxyl terminus termed δX .

IMMUNOGLOBIN molecules exist in two forms: secreted (s) and membrane (m). Secreted molecules make up the neutralizing antibody population in the circulation and secretory fluids, whereas membrane immunoglobulin molecules function as antigen specific receptors on lymphocytes^{1,2}. The majority of mature B lymphocytes strongly express both IgM and IgD on the cell surface with the same idiotype and ligand specificity, and hence, the same variable region³. When the B cell differentiates into a plasma cell upon receipt of an antigen dependent triggering, this same variable region is used for secreted antibody^{4,5}. Although the B cell population expresses surface IgM and IgD about equally⁶, the level of secretory IgD in plasma is about 1,000-fold lower than IgM^{7,8}. This may be due in part to the lower stability of IgD but it also must reflect the fact that cells committed to secretion of IgD must either be quite rare or must secrete δ in small amounts.

The simultaneous expression of two receptors on the B cell surface has led to several theories regarding their differential function. One proposal suggests that membrane IgD may deliver a triggering signal to the B-cell interior whereas membrane IgM provides a tolerizing signal⁹. Another hypothesis holds that membrane IgM conveys a proliferative signal whereas membrane IgD provides a differentiative signal that allows the stimulated B cell to mature to an immunoglobulin secreting plasma cell¹⁰. Yet another hypothesis holds that an IgD signal be required specifically when T cells are involved in B-cell triggering¹⁰⁻¹². Finally it has been proposed that the signal delivered by IgD may be the same as for IgM¹³, in which case the existence of two receptors might serve to improve antigen binding, for example, to antigens with differing epitope density¹⁴. It is clear that membrane IgM and IgD are not completely interchangeable, for the effect of suppression of IgM bearing cells is a profound immune deficiency whereas suppression for IgD has only minor effects^{15,16}.

Both secreted and membrane immunoglobulins have the same basic structure, but they differ in the amino acid sequence at the carboxyl terminus of the heavy chain. Studies by Rogers *et al.*¹⁷ and Early *et al.*¹⁸ on the structure of the messenger RNAs coding for secreted and membrane IgM show that the 3' ends of secreted and membrane mRNAs code for alternative carboxyl terminal amino acids and that these two types of mRNA could result from alternative processing of primary transcripts generated from a single C_H gene. The membrane form of the μ chain has a 41 amino acid hydrophobic carboxyl terminus encoded from two exons located some 1.8 kilobase pairs (kbp) 3' of the C_μ structural gene¹⁸. The secreted form of μ chain bears a shorter hydrophilic carboxyl terminus coded adjacently to the exon coding for the terminal domain, $C_{\mu 4}$.

Both the size and sequence of the two μ mRNAs have been correlated with protein structure data for membrane μ ¹⁹ and secreted μ ²⁰.

In the case of IgD, there is also evidence for alternative carboxyl termini and multiple mRNAs. We initially characterized four C δ exons based on fine structure mapping and nucleotide sequencing of a cDNA clone derived from RNA of the IgD secreting plasmacytoma, TEPC 1017²¹. We also examined C δ clones of mouse genomic DNA. We found that a distally located exon (DC) encodes a hydrophilic segment which is transcribed as the 3' terminus of the major 1.75 kb δ mRNA of the tumour. Dildrop and Beyreuther²² have shown by amino acid sequence analysis of the mouse δ chain that the carboxyl terminal amino acid sequence of the IgD secreted by the hybridoma B1-8.81 corresponds to the DC encoded sequence (see below). Later we²³ and Moore *et al.*²⁴ noted the presence of an additional expressed region approximately 1 kbp 3' of the DC exon. We later showed²⁵ that this region hybridized to two larger (2.9 and 2.1 kb) δ chain mRNAs. These species were minor components of the plasmacytoma δ RNA but major components of spleen δ RNA. We proposed that this general region of the genome, termed VDC (or very distally coded), encodes the membrane carboxyl terminus. Maki *et al.*²⁶ obtained more extensive results and proposed a specific pattern of exons for the 3' end of δ membrane mRNA. Their study was based on the analysis of RNA from hamster-mouse and switch variant hybridomas by examining r-loops between RNA molecules and genomic δ DNA clones. In an IgD secreting hybridoma they found RNA similar in structure to the TEPC 1017 secretory form with the DC terminus. Additionally in both cell types they found membrane form RNA containing sequences from the VDC area with two types of 3' terminal exonic arrangements.

In this article we present a complete DNA sequence analysis of carboxy terminal δ exons correlated with a parallel RNA hybridization study of expression using normal spleen and plasmacytoma RNA as probes. On the basis of these results and those of Maki *et al.*²⁶ and the accompanying results of Fitzmaurice *et al.*²⁷ we present a specific proposal for the structure and RNA splicing patterns of δ mRNA.

Topology of the $\mu\delta$ area

Figure 1a, b presents a scale map of the entire $\mu\delta$ gene complex showing the four exons of the μ constant region, the two exons of the membrane terminus of μ , the three exons of the δ constant region, and the DC and VDC areas located downstream of C $\delta 3$.

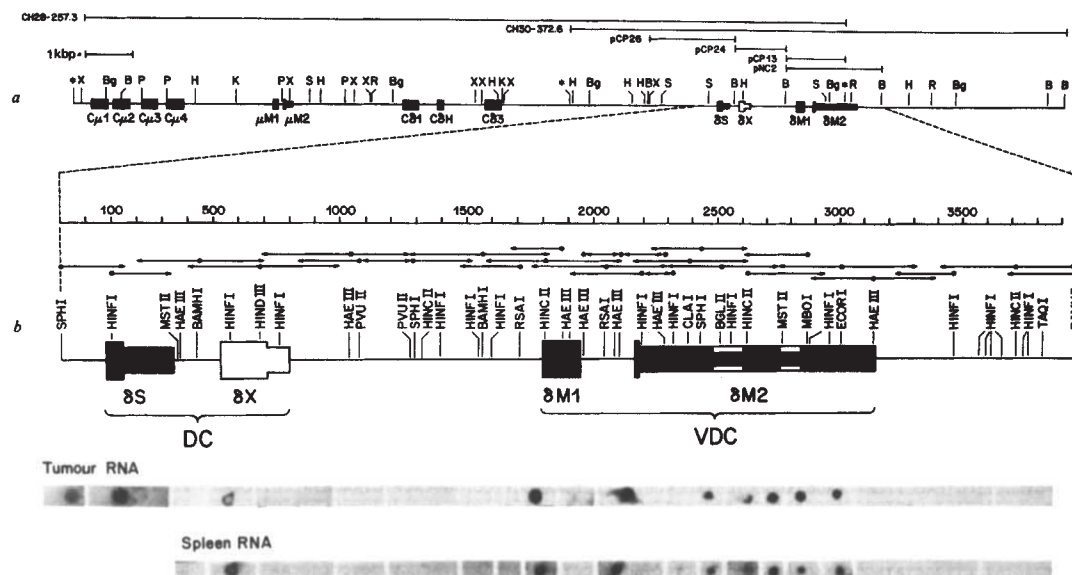


Fig. 1 Map of the $C_{\mu}\delta$ gene complex. *a*, Restriction map of DNA coding for μ and δ genes is shown. The lines at the top indicate the genomic and pBR322 subclones used for sequence analysis. The exons are indicated by black boxes. Location of all C_{μ} exons and the structural exons of $C\delta$ ($C\delta 1$, $C\delta H$, and $C\delta 3$) in CH28-257.3 DNA were previously established^{21,28}. Asterisks represent end points of clones at *Mbo*I sites. Other sites are abbreviated as follows: X, *Xba*I; Bg, *Bgl*II; B, *Bam*HI; P, *Pst*I; H, *Hind*III; K, *Kpn*I; S, *Sph*I; R, *Eco*RI. *b*, Restriction map of sequenced region spanning δ secreted (S) and membrane (M) exons. Boxes denote exons with the raised portion corresponding to sequences that are probably translated into amino acids. The lowered portions of boxes correspond to 3' untranslated regions. The strategy for sequencing by the chemical degradation method²⁹ is denoted by the dots and arrows: Fragments were cleaved and labelled at the dots either on their 5' or 3' ends, and the corresponding horizontal arrows give the direction and length of the sequence obtained; double-ended arrows represent sequences of strand-separated fragments. The scale above is given in base pairs. *c*, Localization of exons by dot-blot hybridization. Total RNA was isolated from TEPC 1017 or normal spleen, enriched for poly(A)⁺ mRNA by oligo (dT) cellulose chromatography and fractionated for size on sucrose gradients. These mRNA fractions were labelled with ³²P by polynucleotide kinase and hybridized to DC and VDC region DNA fragments immobilized on nitrocellulose according to Thomas³⁰. Autoradiographic results obtained from the critical experimental fragments are displayed beneath the corresponding map positions. The length of the fragments is indicated by vertical lines between the spots.

Two λ phage clones, Charon 28-257.3 and Charon 30-372.6 containing the δ region were isolated from shotgun collections of mouse liver DNA. Subsegments were cloned into plasmids for sequencing by the Maxam-Gilbert method²⁹. The endpoints of the various clones are indicated in Fig. 1*a*. An expanded map of the sequenced area is presented in Fig. 1*b* with the sequencing strategy delineated by arrows. The sequence covers approximately 4,100 base pairs (bp), most of which have been sequenced from both strands. Figure 2 shows the complete sequence including the translation into protein of regions that may be expressed, as well as other sites of interest.

Identification of δ exons

The pattern of expression of the sequenced area has been studied by three approaches.

(1) Radioactive RNA from spleen and IgD secreting plasmacytomas was used as a probe for hybridization to DNA fragments immobilized on filters (Dot-blot method³⁰). These results are presented in Fig. 1*c*.

(2) Radioactive fragments from genomic DNA clones were used as probes to detect mRNA species from spleen and tumours by the RNA blot hybridization method³¹. These results are presented by Fitzmaurice *et al.*²⁷.

(3) r-loop data presented by Maki *et al.*²⁶ were scaled up by 10% and superimposed on the sequence with 3' termini aligned with poly(A) addition sites.

As shown in Fig. 1, the sequenced region displays two major zones of hybridization corresponding to the DC and VDC regions. There is no evidence for expression of mature δ mRNA within the approximately 1,000 bp separating these zones, nor 3' to the VDC region, nor 5' between the DC and the $C\delta 3$ domain.

There are two potential exons in the DC area, which are termed δS and δX . The carboxyl terminus of δ secreted by tumours is coded by δS . The region denoted δX seems to be strongly expressed at the RNA level in dot-blot experiments

(Fig. 1*c*). In the VDC area we find two exons, $\delta M1$ and $\delta M2$, which encode the membrane carboxyl terminus of δ . An intervening sequence occurs in $\delta M2$ in some RNA molecules (Fig. 1*c* and ref. 26). However, the position of the intervening sequence as measured from r-loops disagrees somewhat with our conclusions based on dot blots. We will discuss each of these exons in turn.

Carboxyl terminal exon of tumour-secreted δ

The carboxyl terminal 21 amino acids of the δ chain produced by myeloma and hybridoma tumours are encoded on the exon located 4.7 kbp downstream of the terminal structural domain, $C\delta 3$. As detailed in the legend to Fig. 2, the genomic DNA sequence differs slightly from the TEPC 1017 cDNA sequence that we published previously²¹ due probably to a reverse transcription artefact. The actual B1-8.81 protein sequence is identical to that which would be coded from the genomic sequence. It is not homologous to the secreted terminus of either μ ²⁰ or α ³².

Within 10–30 nucleotides 5' to the 3' terminal poly(A) tail of all eukaryotic mRNAs, the sequence AAUAAA³³ and, in rare cases AAUAAA^{34,35}, occurs. An AATAAA poly(A) site which presumably signals the 3' end of δ s mRNA is located 163 bp downstream of the end of the protein. Assuming the actual end of the message is about 20 bp beyond the poly(A) addition site, that 200 A residues are added as a 3' tail and that a typical V region plus leader with 50 nucleotides of 5' untranslated region is present, the calculated length of the full δ s mRNA would be about 1,650 bases. This is in acceptable agreement with estimates of 1,700 to 1,800 bases by gel measurement^{23–27,36}. Likewise, the molecular weight for the secreted, nonglycosylated polypeptide chain calculated from the predicted amino acid sequence coded by the mRNA is 43,800. This is within 1% (ref. 36) and 7% (ref. 26) of reported

TCAGTAATACAGATACAAATGATTGATAACCAAGACAGAGACAGTACATGGGATTAGACCTGAGGTACGGAAAGTTGGGAAGCATTATAGTGTG 100
 CCTTGCTACAGAAAGTCCAGAGCTTTGACAGGTTGAGCAGATTCTAACCTCCCCATGGCAAGCTCTTCAAGGCTGCTTCTGCTACTAGAGAACCC 200
 4S: \ C Y H L L P E S D G P S R R
 TCTTTCCAGCCACCTGCCACTTTATCACTTATCTAGTTTTCCCATGATTCTAGGATGCTACCACCTCTGCTGAGTCAGACGGTCTTCTAGGA 300
 P D G P A L A
 GACCTGATGGTCTGCTGCTGCTGAGACCTTTCTAGGCTGAATGGTTCATCATGTCCACGGTCTGGTTAGCTTGGTCTGCTGTTAAAGTGGTTCAT 400
 ACTACATGTAGCAAGGAAGTTACCTTTCCACTCTCAGGACACTGTAAGAGACACTTCTGTAATATTATAGTGAGAAACGATCAATGAAAT 500
 GAACAAACATAAACCTCTCTTATCACTTACGGGACATTGGCCCTAAGGCTCTTGAGGACGGCTGAACCTGAGTGTCTTAAAGCCATGTGTCACCTCA 600
 GTCAATTAACCTGTGGCTGAGGTGATCTGACTGACAAACAGACAGGGCTGTAGACAGCTTGAAGCTGGACAAAGAGGTTGACCTTCCAGGCAAG 700
 4X: \ D F L F K I Y S K S Y K I S A R T S
 CAAGAGCCTATCACAATTGTTCCAAAGTGTGTGATTAGATCAGAAATTTCTGTTTAAATATACCTCAAAGCTATATAATATCAGCCAGGACTAGT 800
 H K A
 CATAAAGCTTAATCTGAG 900
 TATGATATGTGTATATTTGGTGTGTTGGGTGTGAGATTCCCTGGGAAATTTATAAACAGTTATAGAGCTGCAAGTAGGCTTCCAATGCTTGTCTTC 1000
 CACCATAGGTAATGAACTGGAAGTACAGGTAAGAGAGGCTGCCAAGTGTCTTATTATGCTAAGATTCAGTGTCTCAACAATATTCACATGATTTC 1100
 CTTTGATATGGTGAATGCTTTAGGCAACAGCATCCACAGACTTCACAGTACCCGAGCTGAGCTTACATATGTTCAACAGCAGCTTGTGTAATGA 1200
 TACCAAGCAGCCCTGTGACCCATGGTGTCTTCTTAATCTTCCCATCTGCTGCTGAGCTTTCACACCTTGAAGTTGAGATGTGCTTCTGTAGAAATGA 1300
 TTTCCCTAGGTCGTACATCAGCCACACCTTTCTGATCTTGGACCGTACTTCAGTCTCCAGCTGGGATATTCAATGATGCTTATGATATGGCATAAC 1400
 CATCGTTAAAGCTCTTCCGTTACCTTTAGGATTAAGTTACCGTAGCTAGGATGCTTCCCAATCTCTCCCTCAATTTGCAAGGCTCAGGCTTCTCTGAC 1500
 TCTGGAGTAGTACTCTTTCCCAATGAGTGACCATGACCACTATTCTTCTAAGATGTTACTCCCTTTCTCTGCTCTTTTCATGGCACAATCTTTCTGA 1600
 GAAACCTACCTGCTGCTGCTCTTCTGGGTCTTCTTAAAGTAGTAACCTAATCTGCAACATCCGTGGGACATGATAGATCCACATCAGGTTGT 1700
 TCTCAGATATTCAATCCCTGACAGCACTTCTGTTGAGATTCCAGGATCCAGGCTGGGACATGGTTGAGGGTAGGATCTTTGGGGCAAA 1800
 GTGGTTAGTAGGCACTTGGGACTGAAAGTGAATAGTATTAGCGGAGGAGGGGATATGATCAAAACATTCGTCTGGATGTGAAGCTGTACCTTCAGT 1900
 4M1: \ I V N T I Q
 TCAGTGTTCAGGAGACAGGTTAATAAGAAATTTCTTCTGAGGTTGGAGACCTTCTCATGAGCACTAGTCTTCCCTAGGCACTAGTCAACACCATCC 2000
 H S I M D E L M D S Y M D L E E E N G L F V I A L F L
 AACACTCGTGTATCAGTATGAGCAAGTACAGCTACATGGAATAGGAGGAGAGAACGGCTGTGGGCCCAATGTGACCTTCGTGGCCCTCTTCTCT 2100
 L T L L Y S G F V T F I K /
 GCTCAGCTGTCTCAGTGGTCTGCTCAGCTTCTCAAGGTAGGCTCTTACCACCTCTCTGTAACCTCTCTGGGCACATGTAAGATACCTTCCAG 2200
 GGGCATTTGAAACCCATCGGTACTTCTGGAGTGGCTAGGGAATGCACAGTAATGGGAGGCTGAGCTACTTTCAGTCTTCTGGCCCTATGTAGCTC 2300
 4M2: \ V K
 CAGGACTTTCCTGTTCTCAGACACAGCTCTGTATGACTTCAAGCTCTCTGCGAGGTGAAGTAGACAGGACAGGAACTCTGCAACTACAGAG 2400
 AAAAGTGTCTTCCCTCAACATGAAGCACTAAGAGATACCTTCTATGACAGAGAAATGCCAAGGCCCTCTCTCAATACCTGCTCTCCACCTAGACT 2500
 CCGAGACTCAAAATGCCTAGTATCTTGGCTATAGGCAAGCAGGTGTTCTGCTGCTCCAGCCTACTCATGATGCTCCCTGCTCCCCCAAGCCCTC 2600
 3' UT Possibility 1
 ATCATTCATAAGCATGCTGTGGTCTCAACCCACTGCTCTGCTGTAATTTGTTTGTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTT 2700
 GATCTGAGGAAACAGTGAGAGCCACTAGGGAGGGGTATCAGATCCACATGAAGAAATGTTGTCAGTGAATGAAGAACTCTCTCCCACTTAAAC 2800
 3' UT Possibility 2
 TCTCAGTTGACTTCTCACAACATAGCATATACATGACATGTCACATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT 2900
 TGCACTACTACTCAGATGCACATAAATTTACAGCAGAGCCTTAGGTGACAGGAATGGGAGCTGTGGTCTATTGTAATGAAGACCCCATAGGCTCATATAGT 3000
 TGAATGCTTAGTACCAGGAGTGGAACTCTTAAACAGGATCAGAAAGTTCAGAGGCAAGTCTTGTGTAGGAAGTGTGTGCTGAGGACGGGTA 3100
 GGCTTTCAGGTTTCAAAGTTCATGTCAGGACTAGATCTCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 3200
 TCACTGTCATACCACTCCCTGCTCAGCATGATGTAATGAACCTCAACCTGAAAGCTTACAGGCTCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 3300
 TGTGGCTGCTGCTGCTTCTCATAAGCAAGCAGTACAGTACAGTACAGTACAGTACAGTACAGTACAGTACAGTACAGTACAGTACAGTACAGTACAGT 3400
 TATACAGACCTGCTGATGCTGATTTTCCACTCTCCATCAGACATGACAGTACACACTACCTAAGCTGAGATGGGTCAATGAAGGAACACAGT 3500
 AATACGTTGTTGATGAGCAAGTGTGGTGAACCCCTCTGCT 3600
 ATCAGAGATAGGAGATCAAGAGGCTCCTAATTTGGGATTTAGTCTTAAACAGCTAGAAAGCTCCCTACCCAGACACTATGAACAG 3700
 AGCTGTCATAGTGTGAATGAGTCTGTTGGGATGGAACACTAGTAATCTTGTCTTACAGCTTCAAGTATGATGAAAGTACATTTCTG 3800
 ATATTATGATTCCTTGGTTCAAGGATAATTTTGTAGGACCCAGTACCAAGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 3900
 CAGATTCATGTTGATCAACAGATGATAACAGGGGTGATGGGAAGGATCTCAATGCTGAGTTCGATCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 4000
 GTGGAGAGCAGGAGGAGGATACATTAGGAGATACATCTTCCCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 4095

Fig. 2 DNA sequence of the C8 region spanning the secreted and membrane exons. Acceptor RNA splice site junctions are shown as /; donors as \. Positions of introns within the M2 3' untranslated region consistent with the dot hybridization (Fig. 1) (possibility 1) and electron micrographic data²⁶ (possibility 2) are indicated. Poly(A) recognition sequences are shown in lower case. The δ S sequence given differs from the previously published²⁰ tumour cDNA sequence at positions 296 (deletion), 301 (A), and 360 (T) (see text). Putative amino acids coded by expressed regions are displayed directly above the first base of the corresponding codon in single letter code: Phe, F; Leu, L; Ile, I; Met, M; Val, V; Ser, S; Pro, P; Thr, T; Ala, A; Tyr, Y; terminator, ; His, H; Gln, Q; Asn, N; Lys, K; Asp, D; Glu, E; Cys, C; Trp, W; Arg, R; Gly, G.

size measurements of cell-free translation products of δ chain mRNA.

Possible 'mystery' exon downstream of tumour-secreted terminus

Dot-blot hybridization experiments with both spleen and tumour RNA probes revealed a strongly hybridizing sequence about 300 bp 3' of δ S (Fig. 1). The *Bam*H1-*Pvu*II fragment spanning this region (Fig. 2 nucleotide position 436 to 1,158) also hybridized in RNA blot experiments²⁷ to a minor 2.65 kb RNA present in TEPC 1017. Inspection of the DNA sequence of this region shows that it has signals which might be expected for an expressed carboxyl terminal exon: a consensus acceptor RNA splice site, an open reading frame, a termination codon and a poly(A) addition site. We term this potential exon C δ X. It would encode a 21 amino acid hydrophilic peptide that might serve as an alternative secreted form.

A dominant feature of the δ X region is a tandem repeat (GA)₁₆ followed shortly by (GT)₆(AT)₄ located between the presumed translation terminator and the poly(A) addition site. This repetitive DNA segment in C δ X may be involved in establishment of secondary structure in δ mRNA. A complementary repeat occurs within the intron separating the C δ H and C δ 3 domains³⁸ and again in the intervening sequence between the C μ M2 exon and the C δ 1 domain^{28,38} (Fig. 1). These three sequences could interact to form a complex overall folding of the mRNA. Part of this presumed secondary structure was seen previously when clone 257.3 was denatured and examined on electron microscope grids^{23,28}. Such structures may influence the splicing efficiency of δ S mRNA in normal B cells.

Transmembrane segment of δ

Examination of the DNA sequence near the beginning of the strongly expressed portion of the VDC region reveals an area of strong homology with the μ gene exon, μ M1, which apparently codes for the transmembrane portion of the IgM

receptor molecule^{17,18}. Figure 3 shows an alignment of μ and δ gene sequences in that area. In the 120 bp region coding for the most hydrophobic segments of the μ protein, the μ and δ nucleic acid sequences are over 60% homologous. Allowing for a two amino acid gap, 50% of the amino acids match and similar amino acids are substituted at corresponding positions. We conclude that this segment, which we term δ M1, serves to bind IgD to the membrane. The acceptor splice site beginning the δ M1 exon appears to be somewhat upstream of the corresponding site for μ M1, and the amino acid sequence for δ is thus longer by 14 amino acids than for μ . This segment is not particularly hydrophobic and would probably lie on the outside of the membrane. We term this sequence of the protein the 'spacer'. Interestingly, a cystine occurs in this portion of the IgD protein which might serve in cross-linking IgD half monomers (one H and one L chain) to one another on the membrane as was suggested by Eidels³⁹ and Mescher and Pollack⁴⁰.

Cytoplasmic segment of δ

The δ M1 exon has a donor splice site at a position corresponding exactly to the end of the μ M1 exon. Although this could be used to splice to any of a number of acceptor sites further downstream or not used at all, the next exon according to our hybridization results (Fig. 1c), is a region beginning 220 bp further downstream. This area is homologous to the μ M2 exon and splicing to this acceptor would lead to a carboxyl terminal peptide consisting of just two residues, valine and lysine. These are precisely the same amino acids that are found in the membrane form of μ . There is very little direct evidence concerning the amino acid structure of membrane IgD. The present DNA sequence predicts a molecular weight of 47,500 for the unglycosylated polypeptide assuming a typical V_H region and leader segment. This length is within 2% of the estimate of 48,500 for *in vitro* translation products of δ membrane mRNA²⁶. This agreement would appear to rule out a cytoplasmic component greater than about 30 amino acids. McCune *et al.*⁴¹ have repor-

[illegible]

Fig. 3 Alignment of μ and δ membrane exons. The DNA sequence of the μ membrane coding region¹⁸ is shown aligned with the corresponding region of δ . Bases that match are repeated between the two sequences. Proteins coded from these DNA sequences are shown above and below using the single letter amino acid abbreviations. Regions that were gapped to bring the two sequences into register are shown with dotted lines. RNA splice sites are shown as slashes.

ted that for the human membrane δ chain no large cytoplasmic domain could be demonstrated by proteolytic digestion of *in vitro* products translated in a microsomal system. This is also consistent with the existence of only a small cytoplasmic protrusion.

Beyond $\delta M1$ there are about 15 potential acceptor sites other than $\delta M2$, each of which, if used, would lead to a different carboxyl terminal peptide sequence. Moreover, a donor splice site occurs at the end of the $\delta M2$ exon coinciding with the protein terminator codon so that an array of additional peptides conceivably could be coded beyond the Val-Lys sequence. Since the number of possible mRNAs is quite large, a direct analysis of the membrane δ carboxyl termini at the protein level and analysis of many cloned mRNAs will be needed to establish whether any of the alternative patterns are used. This would be quite difficult if such an event occurs rarely or only during transient states of cellular differentiation.

3' End heterogeneity of δ membrane mRNAs

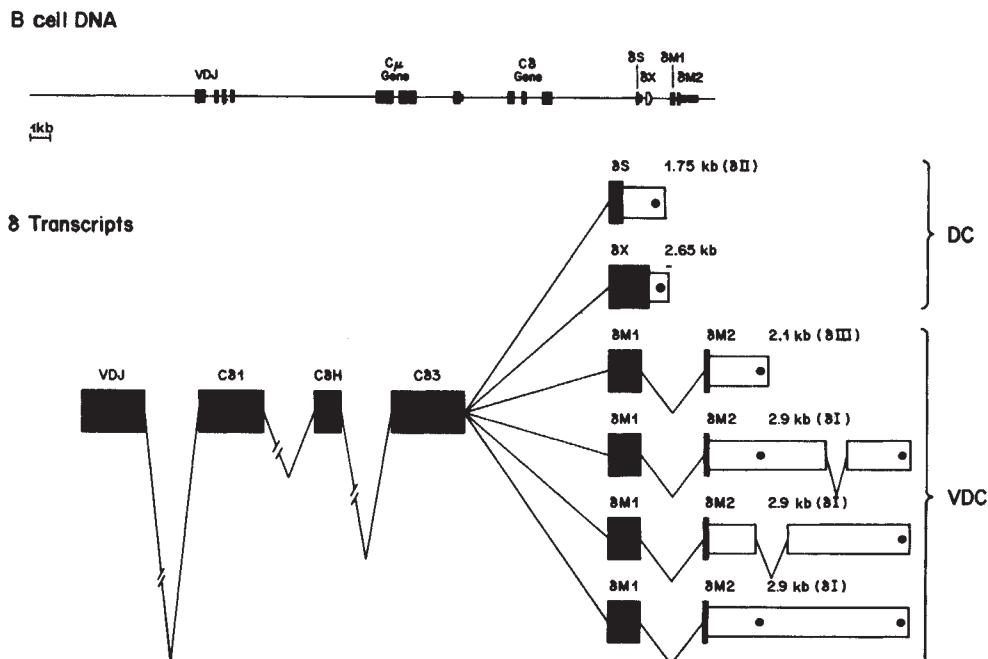
There are two positions at which poly(A) addition sequences occur which could signal the end of the mRNA for membrane

forms of IgD. These are an AATAAA located at nucleotide position 2,680 and an AATTAAA located at position 3,271 (indicated in lower case in Fig. 2). To ascertain whether these are the sites that are in fact used *in vivo*, two restriction fragments from the region were used as probes to examine mRNA by the RNA blot technique. The first fragment from the *Cla* site at 2,568 to the *Mbo*I site at 3,035 covered the region between poly(A) addition sites. This DNA hybridized only to the 2.9 kb δ mRNA (ref. 27, Fig. 2). The *Bam*HI (1,748) to *Cla*I (2,568) fragment derived from the region to the left of both poly(A) sites, hybridized to both the 2.1 kb and the 2.9 mRNA species (ref. 27, Fig. 2). All probes derived from DNA segments located downstream of the *Hae*III site (3,303) were negative on both spleen and plasmacytoma RNA preparations. These results thus show that the 3' ends of the two major forms of membrane δ are correlated with the two poly(A) addition sites we have identified by sequence analysis. The simplest interpretation of these data is that the length difference between the two membrane δ mRNAs results primarily from the poly(A) termination site used, and that there is no difference in the protein coded by these two mRNAs. On this assumption the lengths of the two mRNAs can be calculated as 1,920 and 2,510 bases which is in reasonable agreement with the measured sizes of these RNAs. There is precedence for 3' heterogeneity generating multiple mRNAs which code for the same protein in the mouse dihydrofolate reductase gene⁴². The relative abundances of 2.9 and 2.1 kb forms of RNA²⁷ suggest a strong preference for the more 3' of the poly(A) sites. This may be due to the greater efficiency of AATTAAA as a poly(A) site. Secondary structure may also be responsible for this difference.

RNA splicing within the 3' untranslated region of δ M

The r-loop data of Maki *et al.*²⁶ and the dot-blot data of Fig. 1 both indicate the presence of an intervening sequence near the end of the longer (2.9 kb) message. But the two methods suggest different positions for this intron. A model for the possible ways to express this transcription is presented in Fig. 4. On the basis of a computer search for donor and acceptor splice sites, either position for the intervening sequence could be accommodated. In the case of the r-loop data, a donor acceptor pair at 2,944 and 3,018 could be used (possibility 1 in Fig. 2), whereas a pair at 2,644 and 2,806 could be used to accommodate the dot-blot data (possibility 2 in Fig. 2). The latter possibility would be quite intriguing because it would lead to splicing out of the first poly(A) site from the mRNA

Fig. 4 Proposed RNA processing patterns that generate δ s and δ m RNAs. The upper portion shows a map of the μ d gene complex as arranged in B lymphocyte DNA. The lower portion shows six possible δ mRNA transcripts with alternative carboxyl terminal exons. Regions of the exons (boxes) in black are translated and regions in white are untranslated. Thin lines denote RNA segments that are spliced out in the mature mRNA and black dots denote poly(A) recognition sites. The designations of Maki *et al.*²⁶ for the RNAs are given in parentheses. Note that the same δ amino acid sequence is obtained from all four δ m forms, and that the three 2.9 kb forms differ only in the position (or lack of) the intron in their 3' untranslated region.



which could explain why the second site is used in that message. It is generally thought that polyadenylation precedes RNA splicing⁴³. However, this may not be obligatory in all cases. Thus in δ mRNA the rate of splicing could be rapid relative to that of polyadenylation. Another explanation consistent with our data is that a fraction of the 2.9 kb mRNA is cleaved to the 2.1 kb species and secondarily polyadenylated.

Regulation of δ expression

The μ - δ gene complex is a versatile genetic region which appears to be regulated by almost every known mechanism. Control of mRNA splicing and RNA chain termination are clearly crucial to this regulation, yet almost nothing is known of how this is controlled. Selection of splice sites is precise in the μ δ transcript so that exons of μ never are reassorted with those of δ , yet the alternative carboxyl terminal structures of either chain can be simultaneously expressed or shifted in response to cellular signals. Clearly the point of mRNA termination is an important factor in determining where to splice since it can result in elimination of splice sites from the mRNA precursor molecule. We suggest that secondary structure of the RNA, caused by pairing of the repeated sequences we have observed, may also be an important factor in mRNA splicing since these sites could also be eliminated in response to changes in the poly(A) site selected. Thus a considerable degree of control, both quantitatively and qualitatively, could be exerted by alteration of the overall level of transcription termination enzymes in the B cell, ranging from low in the $\mu^+ \delta^+$ B cell to high in the IgM secreting cell.

A more subtle regulatory effect is suggested by the phasing of RNA splicing in relation to translation. In both μ and δ the splice from the M1 to M2 exon occurs in exact register with the translation phase. This is in contrast to all other splicings known in immunoglobulin mRNAs where the splice occurs after the first base of the codon register. The use of a different phasing relationship for the cytoplasmic side of the membrane may create a barrier to reassortment of interior with exterior domains, since this splicing would create problems of translation reading frame.

Role of secreted forms of IgD

IgD is different from all other immunoglobulins in that both secreted and membrane termini are encoded on separate exons downstream of the body of the C_H gene. Another major difference between IgD and all other Ig's is the lack of a switch-recombination type sequence between μ and δ ⁴⁴ that could efficiently create a secretory genomic arrangement by removing the $C\mu$ gene via the Honjo deletion mechanism⁴⁵. This correlates with the apparent lack of IgD plasma cells in young mice and their clonal appearance in old mice⁷. This suggested to us the possibility that IgD may be principally secreted from resting B cells. Considered in this light the IgD molecule may be more analogous to an antigen specific growth factor than to an ordinary antibody molecule. Clearly some efferent signal from the B cell is needed to account for the fact that in B cell-deficient (anti- μ suppressed) animals, antigen- and idiotype-specific Lyt-1 bearing helper T cells fail to develop⁴⁶. In this role secretion of IgD could serve as a signal to the rest of the immune network, indicating the B cell population level that is expressing each V region and thus IgD may be central to maintenance of B cell homeostasis. The cessation of IgD production when the B cell differentiates either to an

IgM secreting plasma cell (increased RNA termination) or to an IgG or IgA secreting plasma cell (deleting δ) would both be entirely logical from a structural and functional point of view.

Do IgD and IgM deliver different signals?

It was to be expected that the general principle of membrane attachment that was demonstrated for μ would apply also to δ , but it is very interesting that the amino acids coded by δ M2 are identical to those of μ M2. This is the portion of the protein that would presumably extend into the cytoplasm and which could deliver a signal to the cell's interior. Our data predict that the signal delivered to the inside of the cell by δ M2 coded IgD would be identical to that delivered by IgM—at least if the nature of the signal is determined by the terminal amino acid sequence exposed to the cytoplasm. However, it is possible that the entire immunoglobulin may be internalized as a result of antigenic stimulation, or other surface proteins (for example, Fc receptors or Ia molecules) may interact specifically with δ and not μ . In this case the two immunoglobulins could deliver different signals despite the identical nature of the portions presented to the cellular interior.

Conclusions

With the completion of the DNA sequences of the terminal exons presented here, we have determined the complete structure of the murine C δ gene. The gene structure leads us to postulate the existence of three forms of the δ protein: δ S, δ M and δ X. The existence of δ X, however, remains provisional at present since it is based entirely on nucleic acid data. Our conclusions concerning δ transcription are summarized in Fig. 4. At least six mRNA species coding for δ have been discussed which differ only in their 3' ends. Four of them probably code for the same membrane protein (δ M) although additional forms generated by splicing to alternative 3' coding segments cannot be ruled out in lieu of cDNA data.

The gene structure of δ is substantially different from all other immunoglobulins in that the C δ constant region has only two domains²¹ separated by a long, exposed hinge region. This may lead to the instability of IgD in serum, a feature that would be crucial if the secreted molecule (δ S or δ X) is to serve as an idiotype bearing chemical messenger as we suggested above.

In membrane δ , a 26 amino acid spacer region separates the last domain from the membrane anchor. This region is completely different for IgD, IgM and presumably for other classes. It thereby is a class-specific marker that can distinguish the immunoglobulin produced by its particular B cell from other irrelevant immunoglobulins that may be cytophily adsorbed to the surface. In this role the spacer would be a good target for class-specific recognition by T cells or T-cell derived factors that promote maturation and differentiation of the immune response.

As for the antigen-specific receptor functions of IgD compared with those of IgM, the identity of the internal (cytoplasmic) amino acids suggests that the information pertinent to their differential roles does not reside on the inside of the B cell membrane.

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LETTERS TO NATURE

IUE observations of variability and differences in the UV spectra of double quasar 0957+561 A, B

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Since the discovery¹ of the double quasar Q0957+561 A, B by Walsh *et al.*, their proposition that it represents a double image of a single object formed by an intervening galaxy acting as a gravitational lens has been amply confirmed. The intervening galaxy has been detected optically^{2–4} at a redshift of 0.36 (ref. 5). Further, a remarkable similarity in fine structure of the radio images has been demonstrated with VLBI observations⁶ and the required frequency invariance of the intensity ratio has been established from the UV⁷ through the optical⁸ and IR⁹ to the radio¹⁰ regions of its spectrum. The first UV observations of the double quasar were made with IUE in December 1979, and have been supplemented by data obtained during September and December 1980. Here we present these new data together with an analysis based on the images being a gravitational lens effect.

The log of the new observations is given in Table 1. As previously, the nearby star HD237844 (B3, 9.4 mag) was observed as a comparison. This is necessary because there is some overlap of the two spectra of Q0957+561 A and B; these are separated by a deconvolution procedure which uses the profile of the instrumental point-spread function perpendicular to the direction of dispersion derived from the spectrum of the comparison star. The procedures for data extraction, deblending and correction for instrumental sensitivity are described elsewhere⁷.

Because the object is faint (17th mag) for IUE, two separate observations have been made at each epoch and combined to improve the signal-to-noise ratio. For completeness, Fig. 1 covers the previous⁷ as well as the present data and shows the mean of two spectra, in 5 pixel bins ($\sim 11 \text{ \AA}$), of component A (north) and component B (south) in each of the epochs 1979 December, 1980 September and 1980 December. Apart from the continuum, the only spectral features clearly present are the emission and absorption lines of Ly α . Their wavelengths are consistent with the respective redshifts $z_e = 1.41$ and $z_a = 1.39$ given by Walsh *et al.*¹.

From the spectra in Fig. 1, the continuum flux in the range 2,500–2,800 $\text{\AA}$ and the emission flux in the Ly α (+N v) line were determined (see Table 2). It is evident that no changes have occurred in the spectrum of component B; the intensity of its continuum and (Ly α + N v) emission lines are the same, within the errors, at all three epochs. Component A, on the other hand, shows substantial changes. Its continuum level had fallen by a half by 1980 September and had almost recovered

to its 1979 December value in 1980 December. There is similar but less pronounced variation of the intensity of (Ly α + N v).

Within the observational limits, the equivalent widths of the emission lines (Ly α + N v) in both components A and B were equal during December 1979 and December 1980 (see Table 2). During September 1980 the equivalent width of emission line in component B was similar (within observational limits) to equivalent widths in components A and B during December but the equivalent width of line in A had increased in the order of 50% compared with line in B, which was greater than the observational uncertainty. It is unlikely that the observed changes in the line and continuum intensity are due to instrumental effects because at the resolution of IUE, vignetting should affect both line and continuum intensities equally. The changes in the equivalent widths of emission lines also indicate that there is a phase lag between a change in the continuum level and the resultant change in the line intensity. Clearly the time grid is too coarse to measure this phase lag but an upper limit of 3 months can be established. This in turn gives an upper limit of 0.1 pc ($H_0 = 50 \text{ kms}^{-1} \text{ Mpc}^{-1}$) for the distance between the source of continuum radiation and the broad line emission region. This separation is an order of magnitude smaller than the separation assumed for modelling the broad line region¹¹.

The fact that the variation has occurred in A is important because any change in the source will be observed in this component first and should then recur in B after the appropriate time delay. To see what information may be available about this critically important parameter, our UV data have been combined with all other currently published intensity data; these are listed in Table 3 and plotted against time in Fig. 2. The range of frequency covered is very large, observations being made in the radio, IR, optical and UV. Where there is overlap there is reasonable agreement between the different observations. Some of the dispersion can be explained by the fact that the optical data include a contribution from the intervening galaxy which varies with wavelength, but the 1980 January observations⁵ at 4,000 $\text{\AA}$ seem more discrepant than expected from the errors. Over the months April–December 1979, the flux ratio shows no significant change and has the same value, within the errors, in all spectral regions from the radio to the UV. Beyond January 1980, the continuum flux ratio B/A at optical and UV frequencies starts to increase, reaching a maximum during August–September and almost recovers to its intrinsic value by December 1980. Unfortunately only UV observations reported here and available near maximum but the optical data taken immediately before¹² and after¹³ support the existence of the maximum. Table 2 shows clearly that the observed changes are confined to component A, both the continuum flux and (Ly α + N v) emission line flux of component B remaining constant within the error. This could be due to (1) a star in the halo of the lensing galaxy occulting only sight-line A^{14,15}, (2) the lensed quasar being intrinsically variable, a change having propagated along sight-line A but not along B.

If a star in the halo of lensing galaxy occults a sight-line then radiation at all frequencies will be attenuated but line radiation

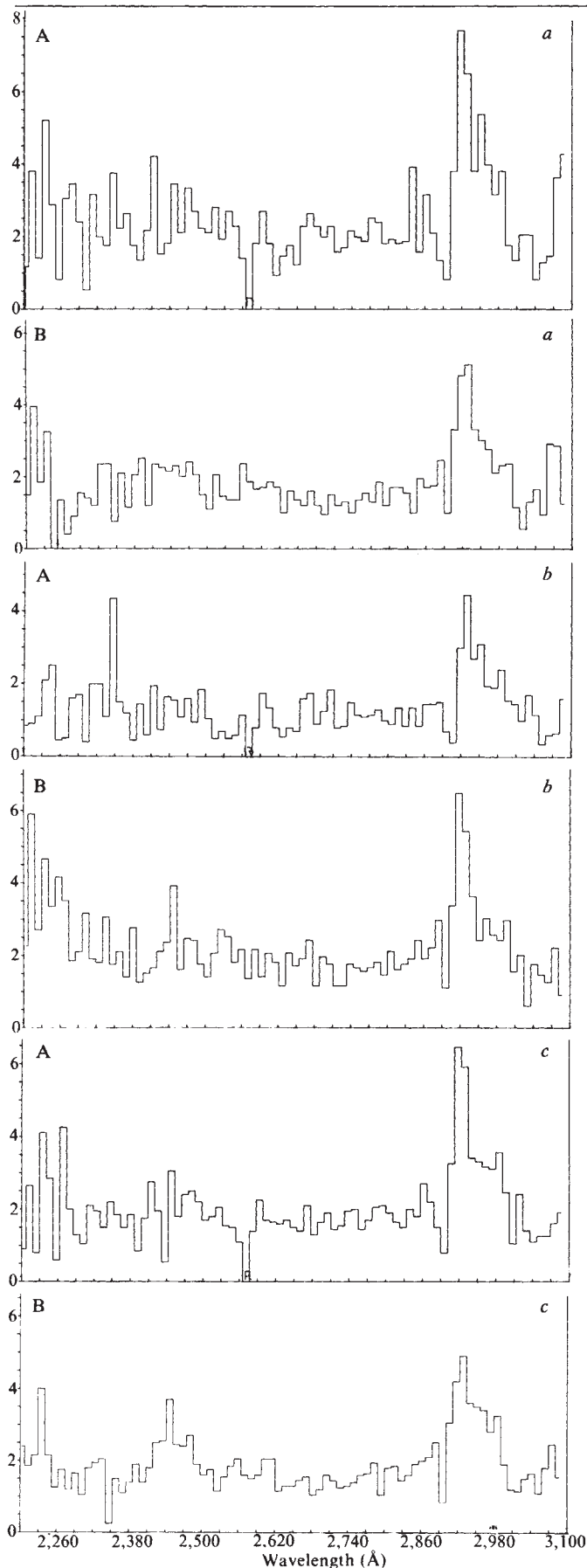


Fig. 1 UV spectra of double quasar 0957+561 A, B in 5-pixel bins (~ 11 Å). *a*, Mean of images LWR 6472/73 obtained on 24–25 December 1979. *b*, Mean of images LWR 8877/96 obtained on 23–26 September 1980. *c*, Mean of images LWR 9445/58 obtained on 6–8 December 1980.

Table 1 IUE observing log of 0957+561 A, B

Object	Image LWR	Integration time (min)	Date
0957+561 A, B	8877	350	23 September 1980
HD237844	8878	2	23 September 1980
0957+561 A, B	8896	305	26 September 1980
HD237844	8897	2	26 September 1980
HD237844	9444	2	6 December 1980
0957+561 A, B	9445	373	6 December 1980
HD237844	9457	2	8 December 1980
0957+561 A, B	9458	376	8 December 1980

will be attenuated less than continuum radiation as the source of line radiation is more extended than the source of continuum radiation¹⁴. At the resolution and signal-to-noise ratio of UV data it is not possible to estimate whether the line radiation has been attenuated more than the continuum radiation. On the other hand, if the lensed quasar is intrinsically variable then UV radiation is more likely to keep in step with visible radiation than with radio emission. The trend in the visible data is very similar to the trend in the UV data. Lacking the crucial radio data for September 1980, we conclude from the present observations that the lensed quasar is intrinsically variable but that during 1979 April–December the entire lens system was stable and no changes were propagating through this system and the observed mean ratio $B/A = 0.74 \pm 0.01$ represents the intrinsic ratio for the lensing system. Around August–September 1980 a change occurred in the lensed quasar and this change has been propagated along sight-line A but had not arrived along sight-line B by 1980 December and this allows us to place a lower limit on the delay time $\Delta T(B-A)$. At the 3σ level, changes have occurred in both the continuum and $(Ly\alpha + N\,V)$ emission fluxes of component A in 1980 early May and are still undetected in B in 1980 December. Hence,

$$\Delta T(B-A) > 0.6 \text{ yr}$$

Current theoretical estimates of the time delay are very uncertain because it depends not only on the mass distribution in the lensing galaxy but also the mass distribution, both visible and hidden, throughout the cluster of which it is the brightest

Table 2 Continuum and emission fluxes and equivalent widths of emission and absorption

Image LWR	Continuum flux ($\times 10^{-15} \text{ erg cm}^{-2} \text{ s}^{-1} \text{ Å}^{-1}$)		
	A	B	B/A
6472/73	2.0 ± 0.1	1.5 ± 0.1	0.75 ± 0.06
8877/96	1.0 ± 0.1	1.7 ± 0.1	1.7 ± 0.2
9445/58	1.7 ± 0.1	1.5 ± 0.1	0.88 ± 0.08
	$(Ly\alpha + N\,V)$ emission flux ($\times 10^{-13} \text{ erg cm}^{-2} \text{ s}^{-1}$)		
	A	B	B/A
6472/73	2.4 ± 0.1	1.8 ± 0.1	0.75 ± 0.05
8877/96	1.5 ± 0.1	1.8 ± 0.1	1.2 ± 0.1
9445/58	2.0 ± 0.1	1.9 ± 0.1	0.95 ± 0.07
	$(Ly\alpha + N\,V)$ emission line equivalent width (Å) (observers frame)		
	A	B	
6472/73	120 ± 6	120 ± 10	
8877/96	150 ± 12	106 ± 8	
9445/58	118 ± 8	127 ± 9	
	$Ly\alpha$ absorption equivalent width (Å) (rest-frame values in brackets)		
	A	B	
6472/73	$22 (9.3)$	$8.4 (3.5)$	
8877/96	$17 (7.0)$	$9.5 (4.0)$	
9445/58	$18 (7.7)$	$10.3 (4.3)$	
Mean EW	$19 (8.0)$	$9.4 (3.9)$	
Mean column density	$1.2 \times 10^{20} \text{ cm}^{-2}$	$2.8 \times 10^{19} \text{ cm}^{-2}$	

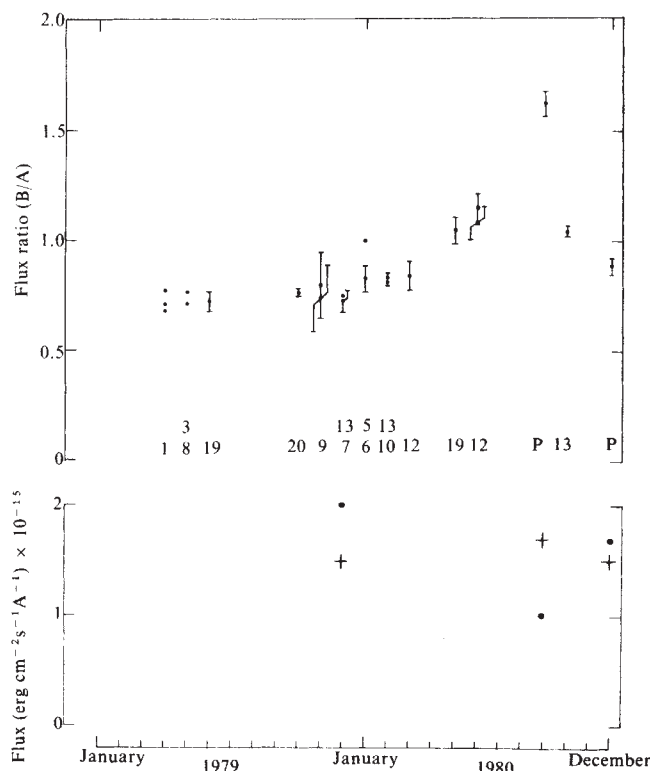


Fig. 2 The variation in the flux ratio QSO(B)/QSO(A) during 1979-80 (upper plot) and the measured UV continuum fluxes (2,500-2,800 Å) from QSO, A(●) and QSO, B(+). The numbers are references from which data were obtained; P, the present data.

member. Hence, from a grid of assumed positions for the cluster centre, Dyer and Roeder¹⁶ obtained time delays in the range 0.03-1.7 yr. With extensive new CCD observations of the surrounding cluster of galaxies and a more detailed modelling, Young *et al.*⁵ are able to fit the data with time delays of up to ~5 yr. Clearly, the observational limit given above restricts the range of possible models and continued monitoring to detect the same event in component B should give an actual measurement of the time delay. Because this scales inversely as the Hubble constant H_0 there is, in principle, the exciting possibility of measuring this out to $z = 1.4$, but uncertainties in the mass distribution of the lensing galaxy and surrounding cluster are too great to make an unambiguous determination of H_0 from a single time delay between two images. Young *et al.*⁵ point out that a good estimate of H_0 might be possible if two time delays, one between image A and B and a second between A or B and as yet unobserved third image, were measured.

The equivalent widths of the Ly α absorption lines occurring at a redshift $z = 1.39$, were also measured in the IUE spectra and are listed in Table 2 with the rest-frame values in brackets. As with the other measurements given in Table 2, these are made from the mean of two spectra taken of each component at three different epochs. These are reproduced in Fig. 3 but, unlike Fig. 1, they are unbinned and only cover the region of the Ly α line. An accurate estimate of the standard error is not possible in this case as a major uncertainty arises from drawing in a continuum in the neighbourhood of the emission component; we judge that the mean value for each component has an accuracy in the range 10-20%. The resulting column densities given in Table 2, derived on the basis of radiation damping, are therefore accurate to ~20-50%.

Clearly the Ly α absorption in component A is greater by a factor of 2 than in B. The separation of the two light paths in the region of absorption is therefore a measure of the spatial structure in the absorption system. If we assume that the absorption system is at a cosmological distance then for $H_0 = 50 \text{ km s}^{-1} \text{ Mpc}^{-1}$, a separation of ~0.5 kpc is obtained for the two absorption regions. The column density $\sim 10^{20} \text{ cm}^{-2}$ for H I

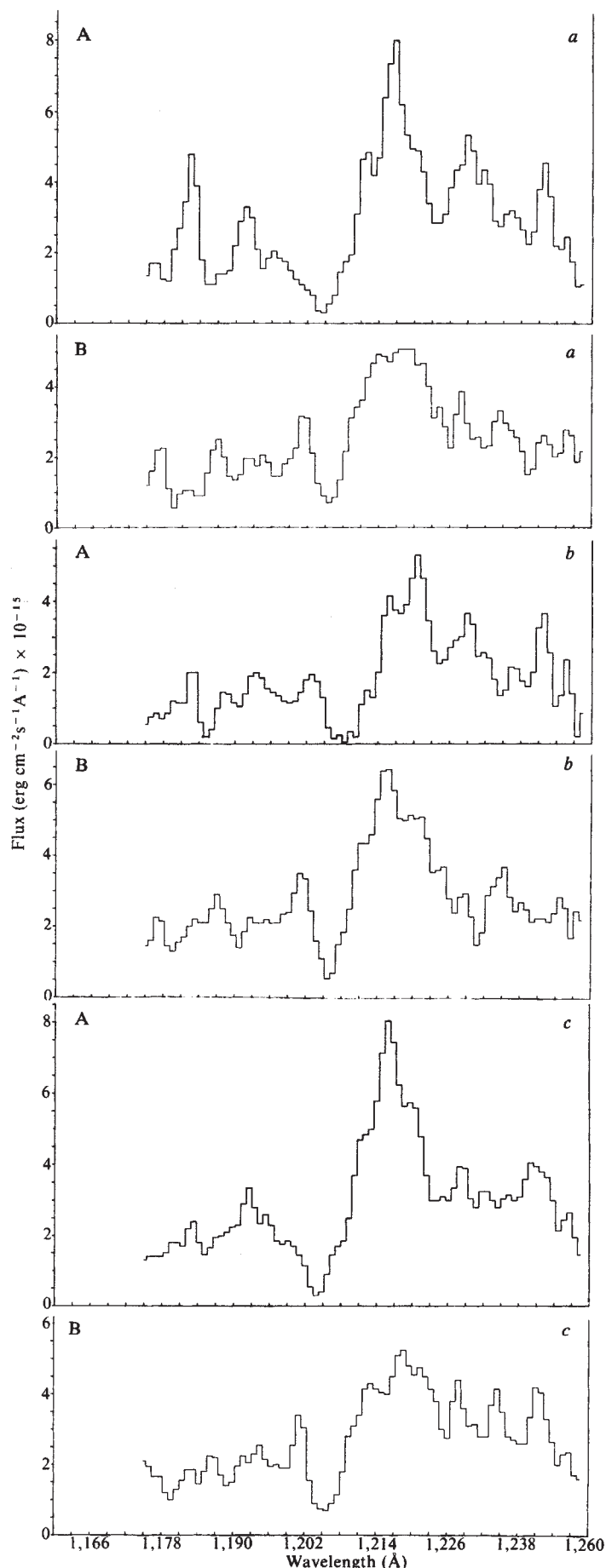


Fig. 3 Mean Ly α emission and absorption line profiles (in rest-frame) in a, December 1979; b, September 1980; c, December 1980.

Table 3 Observed flux ratio QSO(B)/QSO(SA)

Date	λ	B/A	Ref.
April 1979	3,500 Å	0.68	1
April 1979	4,500 Å	0.70	1
April 1979	5,500 Å	0.77	1
May 1979	4,000 Å	0.76	3
May 1979	4,000 Å	0.71	8
June 1979	Radio	0.72 ± 0.04	19
October 1979	Radio	0.76 ± 0.01	20
November 1979	1.65 μm	0.80 ± 0.15	9
November 1979	2.2 μm	0.74 ± 0.15	9
December 1979	U	0.75 ± 0.01	13
December 1979	UV	0.72 ± 0.05	7
January 1980	4,000 Å	1.00	5
January 1980	Radio	0.83 ± 0.06	6
February 1980	Radio	0.81 ± 0.01	10
February 1980	U	0.83 ± 0.01	13
March 1980	V	0.84 ± 0.07	12
May 1980	B	1.05 ± 0.06	19
June 1980	V	1.15 ± 0.07	12
June 1980	V	1.09 ± 0.07	12
September 1980	UV	1.62 ± 0.06	Present data
October 1980	U	1.04 ± 0.02	13
December 1980	UV	0.88 ± 0.04	Present data

in this region is higher than the values obtained for the halo of our Galaxy ($\sim 10^{19} \text{ cm}^{-2}$)¹⁷. If, however, the absorption system is intrinsic to the QSO and represents remnants of ejecta from the quasar then an upper limit to its distance from the QSO can be established by assuming that it has been moving at its relative velocity of $\sim 1,900 \text{ km s}^{-1}$ for $\sim 10^6 \text{ yr}$, the longest kind of lifetime expected for quasars¹⁸. This gives an upper limit on distance of $\sim 2 \text{ kpc}$, which in turn gives an upper limit to the separations of the two absorption regions of $\sim 10^{17} \text{ cm}$.

We thank the staff of the IUE Observatory (VILSPA) in Spain for assistance and hospitality.

Following the submission of this paper, two reports^{21,22} of ground-based photographic photometry of the double quasar have appeared. Within the errors the intensity ratios B/A given in these papers agree with our results, that is they show a gradual increase up to June 1980 and a decrease from October 1980. (The maximum departure as revealed by IUE occurred during the intervening period when the double quasar was inaccessible to ground-based optical observations.) However, the two sets of optical data^{21,22} indicate that component A remained constant and that B varied, whereas our results indicate the exact opposite.

We believe that only further observations will resolve this contradiction. For any reasonable lens model for the double quasar⁵, events occurring in component A will be followed by similar events in B. Thus the optical observations indicate that variations in A must have occurred in the past, while our results indicate a forthcoming variation in B. The various lower limits on the delay time deduced from the respective data will obviously depend on which data are confirmed. We can predict that the UV and optical intensity of B will fade by a factor of ~ 2 over a period of months. This is best tested by continuing to monitor the object in the optical and UV; if positive, it will give an unambiguous measurement of the delay time.

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Asteroid rotation rates depend on diameter and type

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The rotational frequency of main-belt asteroids is shown here to depend on both asteroidal type and diameter. If asteroids of any one diameter are considered, then, on average, M asteroids rotate faster than S asteroids which in turn rotate faster than C asteroids. This shows that asteroids which have been classified by their surface properties alone have different bulk properties. For all three types, although the dispersions of the frequencies are large, we prove that the mean frequency increases linearly with the mean diameter. In both the C and S plots of mean rotational frequency against mean diameter there are discontinuities at diameters $\sim 125 \text{ km}$ and $\sim 105 \text{ km}$, respectively, which may differentiate primordial asteroids from their collisional products.

Rotational frequencies of asteroids have been controversial ever since Alfvén¹ pointed out that all bodies except the Sun in the Solar System that have not been affected by tidal braking display an approximate isochronism of spin. Publication of opinions on the dependence of rotational frequency on asteroid diameter and type has since kept pace with the growing data.

McAdoo and Burns², for example, using a sample of 64 asteroids, claimed that smaller asteroids rotate more rapidly than larger ones. More recently, using a sample of 182 asteroids, Harris and Burns³ also concluded that there is a tendency for the smaller asteroids to rotate faster than the larger ones. However, they also concluded that there is little change in rotation rate with size when either C- or S-type asteroids alone were considered and that C-type asteroids appear to rotate $\sim 80\%$ less rapidly than the other asteroids. They consider that this, together with observational selection effects, could account for the apparent size dependence of the frequency distribution of the sample as a whole. (Various surface properties are used to separate asteroids into a small number of distinct groups. The chief difference between C and S asteroids is their albedos: C asteroids are much darker than S asteroids. For a complete discussion of the CSM asteroid classification scheme see ref. 9.)

Tedesco and Zappala⁴ used data on 321 asteroids to define a set of 134 main-belt asteroids having reliable, photoelectrically determined rotational periods. Their criteria for inclusion in the set were designed to minimize observational selection effects and to isolate a set of asteroids with similar histories. With this set they were still unable to find any definite relationship between asteroid size and rotation rate but, in contrast to Harris and Burns, they recognized a group of large asteroids (diameter $\geq 175 \text{ km}$) that appear to rotate faster than the rest of the sample. They also found, like Harris and Burns, that S-type asteroids tend to rotate faster than C-type asteroids but concluded that the difference was statistically insignificant.

Burns and Tedesco⁵ concluded that there is no statistically significant size dependence for rotational frequency within either the S or C taxonomic type, but asteroids with diameters $> 175 \text{ km}$ do rotate faster than the rest. They also consider that a much larger data base is needed if any trends that do exist are to be discerned. We now show that this is not the

case. By analysing the same data set as that used by Tedesco and Zappala⁴ we show that trends do exist in the data.

The data are shown in Fig. 1a: any trend is not apparent to the eye, but there is a significant linear correlation between rotational frequency and diameter. By ordering the chosen population in diameter and calculating running means, we can plot the mean rotational frequency against the mean diameter (Fig. 2a). In Fig. 2a the running box always contains 19 ($=n$) asteroids and was shifted through the chosen population one asteroid at a time.

The central limit theorem (see, for example, ref. 6) allows us to relate σ , the standard deviation of the distribution of the means of the samples, to σ_p , the standard deviation of the chosen population by

$$\sigma = \frac{\sigma_p}{\sqrt{n}} f(N, n) \quad (1)$$

where n is the number of objects in each sample taken from a population of N objects, and

$$f(N, n) = \left(\frac{N-n}{N-1} \right)^{1/2} \quad (2)$$

is a correction factor which allows for the fact that sampling

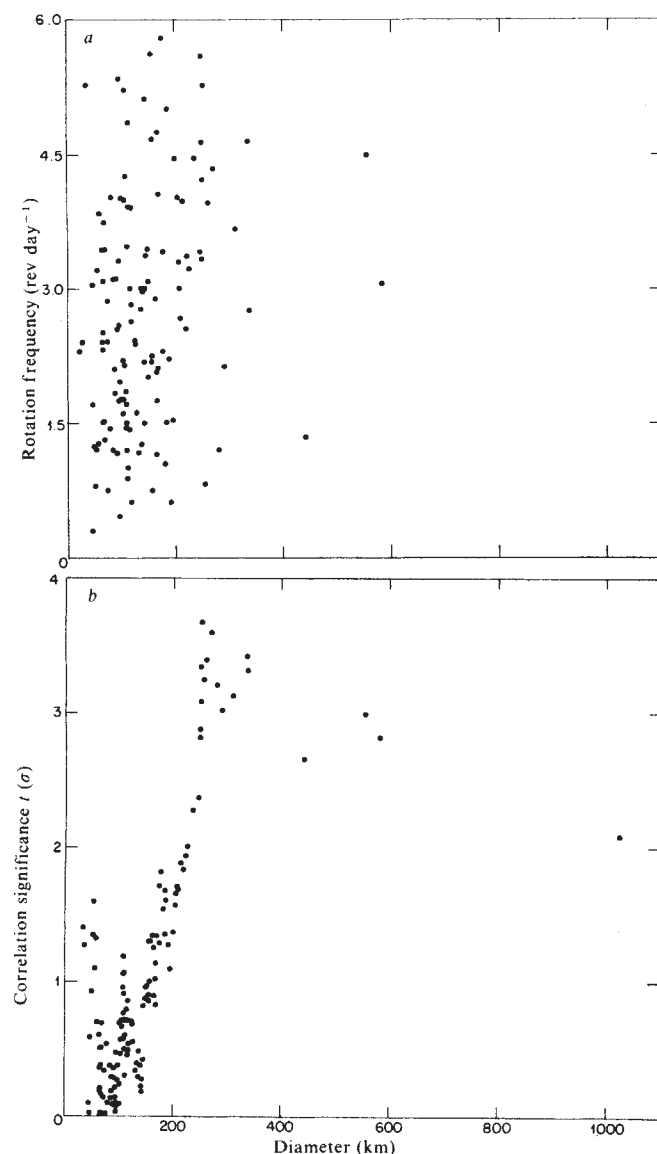


Fig. 1 a, Rotational frequency and diameters of 134 main-belt asteroids with reliable photoelectrically determined rotational periods. All data are from ref. 4. b, Variation of the statistical significance (σ) of the linear correlation coefficient with increasing diameter for the data shown in a. Note that the correlation is greatest for the 130 asteroids with diameters <400 km.

takes place without replacement: the first asteroid in the sample is chosen from N asteroids, whereas the n th is chosen from $N-n+1$.

Figure 2a shows clearly that rotational frequency increases with asteroid diameter. The mean rotational frequency increases linearly from a minimum of 2.1 rev day⁻¹ (period = 11.1 h) for asteroids of diameter 60 km to a maximum of 3.7 rev day⁻¹ (period = 6.5 h) for asteroids of diameter 230 km, that is, the mean frequency increases linearly from a point 2.0 σ below the mean to one 3.5 σ above.

The running box method gives a good description of the dependence of rotational frequency on diameter, but with this method it must be remembered that because each box contains 19 ($=n$) asteroids from a population of 134 ($=N$), then there are only 7 ($=N/n$) independent groups of data. Plots of the mean frequencies and the mean diameters of such independent groups for various values of n (19, 11 and 7) are shown in Fig. 2b, c, d. The uncertainties in the mean frequencies of these groups were estimated from the sample variances and in each case they compare well with the expected uncertainties estimated from the variance of the population as a whole.

We do not find any evidence of a discontinuity at diameter 175 km. Any trend that exists in the data seems to be present throughout the whole population. There is a suggestion, however, that mean rotational frequency does not increase indefinitely with mean diameter, but reaches a plateau of ~ 3.5 rev day⁻¹ (period ~ 7 h) at diameter ~ 230 km. However, because the population contains asteroids of different taxonomic types these values are not suitable for direct interpretation.

A useful measure of the significance of the correlation between rotational frequency and diameter is given by the linear correlation coefficient r . The statistical significance of r can be estimated from

$$t = r \left(\frac{N-2}{1-r^2} \right)^{1/2} \quad (3)$$

and if N is large (≥ 100) then the distribution of t can be assumed to be normal⁷ and t then represents the number of standard deviations (σ) from the mean. Figure 1b shows the correlation significance t as a function of increasing diameter D' . That is, the asteroids were ordered in diameter and r and t were calculated for those asteroids with diameter $\leq D'$. We find that t increases steadily with increasing D' to some value $>3\sigma$ (the maximum is 3.6σ). The inclusion of the largest four asteroids in our chosen population causes t to decrease. In fact, if all asteroids including Ceres are included then t is reduced to 2.05σ . However, this is entirely consistent with our statement that rotational frequency increases with diameter but a maximum mean frequency is reached at a diameter of ~ 230 km.

The file containing rotational periods in the Tucson Revised Index of Asteroid Data (TRIAD) is maintained by Tedesco⁸. Quality codes are used to indicate the reliability of the quoted data. Quality 1 indicates that the quoted period has been estimated from observations covering less than a complete rotation cycle. Quality ≥ 2 indicates secure results, that is, the rotational period is known to within a fraction of 1 h or better.

The 134 asteroids defined by Tedesco and Zappala⁴ are all of quality ≥ 1 , and include 48 S-, 45 C- and 9 M-type asteroids. Data describing the frequency (ω) and diameter (D) distributions, that is, the mean rotational frequency $\langle \omega \rangle$, the mean diameter $\langle D \rangle$ and the standard deviations σ_p of each subset of M-, S- and C-type asteroids are given in Table 1. Table 1 also contains data for those asteroids in the set of 134 with rotational periods of quality ≥ 2 . Running box plots of the data in Fig. 3a show that the tendency for mean rotational frequency to increase with diameter, which we have proved to exist in the data set as a whole, is not determined by the data in any one subset but is equally present in all three subsets of M-, S- and C-type asteroids: the tendency is a characteristic common to all major asteroidal types. Running box plots for the better quality data (quality ≥ 2) are shown in Fig. 3b.

Table 1 Asteroid data

Subset	Type	No.	$\langle\omega\rangle$ (rev day ⁻¹)	σ_p (rev day ⁻¹)	$\langle D \rangle$ (km)	σ_p (km)	m	ω_0 (rev day ⁻¹)
1	All	34	2.71	1.33	151.1	117.4	—	—
1	C	45	2.47	1.31	193.6	148.7	0.013	0.24
1	S	48	2.57	1.08	117.5	55.8	0.014	0.73
1	M	9	3.28	1.75	113.6	62.4	0.023	0.63
2	C	25	2.61	1.49	218.4	189.1	0.023	-1.65
2	S	32	2.80	0.98	134.6	54.3	0.023	-0.17
2	M	6	3.85	1.78	128.2	72.6	0.024	1.08

Subsets: 1, photoelectrically observed, main-belt asteroids with rotational periods of quality ≥ 1 listed by Tedesco and Zappalà⁴; 2, as 1 but only asteroids with rotational periods of quality ≥ 2 . These subsets are further divided by taxonomic type.

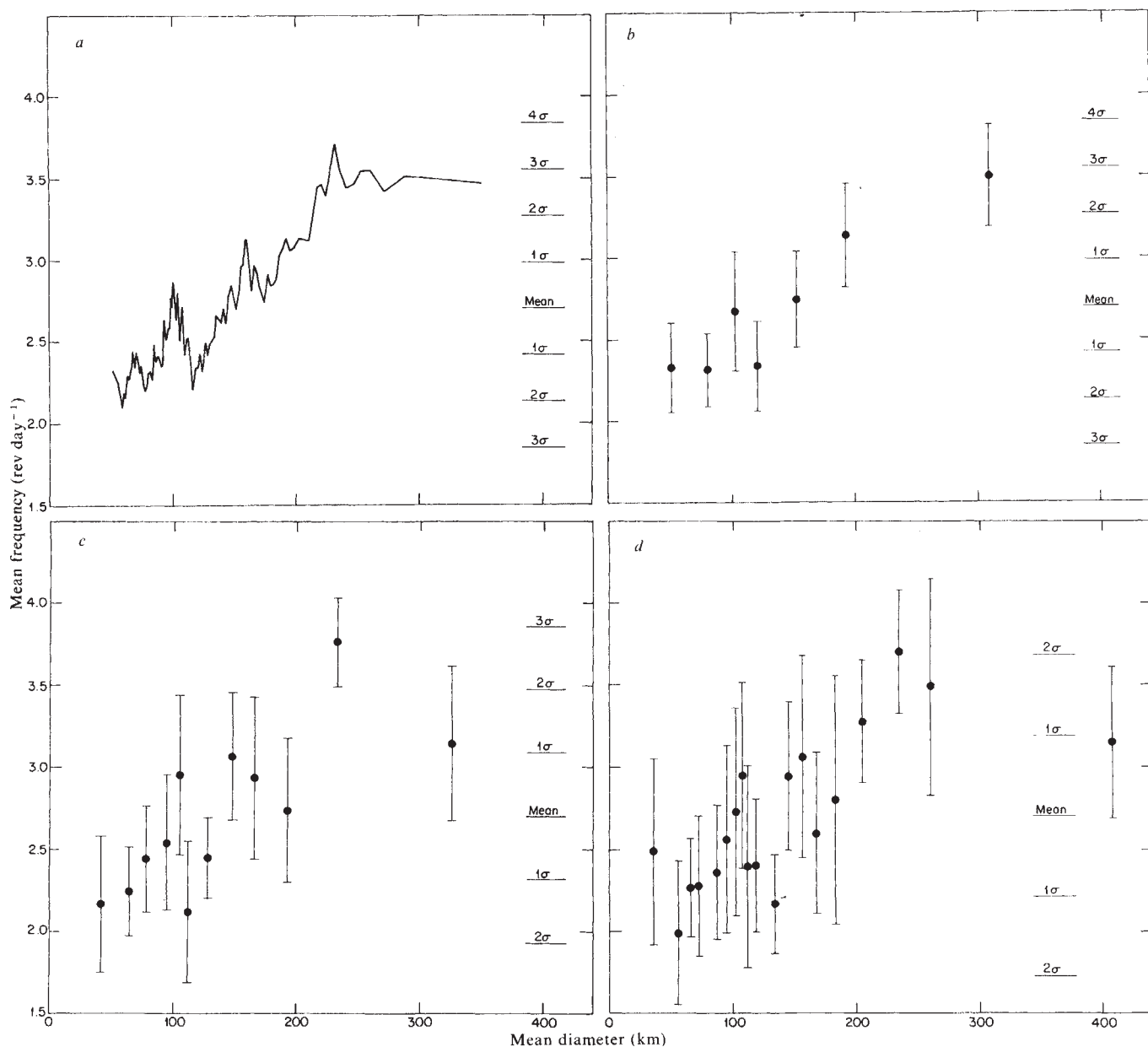


Fig. 2 *a*, A plot of the mean rotational frequency against the mean diameter for the 134 asteroids shown in Fig. 1 obtained by applying a running box method to the chosen population which were sorted in order of increasing diameter. The σ levels are derived by the application of the central limit theorem to the distribution of the mean of the samples of the population. The running box (and hence each sample) contained 19 asteroids and was stepped through the population one asteroid at a time. Mean rotational frequency clearly increases with mean diameter reaching a plateau ~ 3.5 rev day⁻¹ (period ~ 7 h) at mean diameter ~ 240 km. In *b*, *c* and *d* we show plots where the ordered data are shown in independent groups of *b*, 19; *c*, 11; *d*, 7 asteroids. The uncertainties in the mean frequencies were estimated from the sample variances and in each case they compare well with the expected uncertainties (shown at the sides) estimated from the variance of the population as a whole.

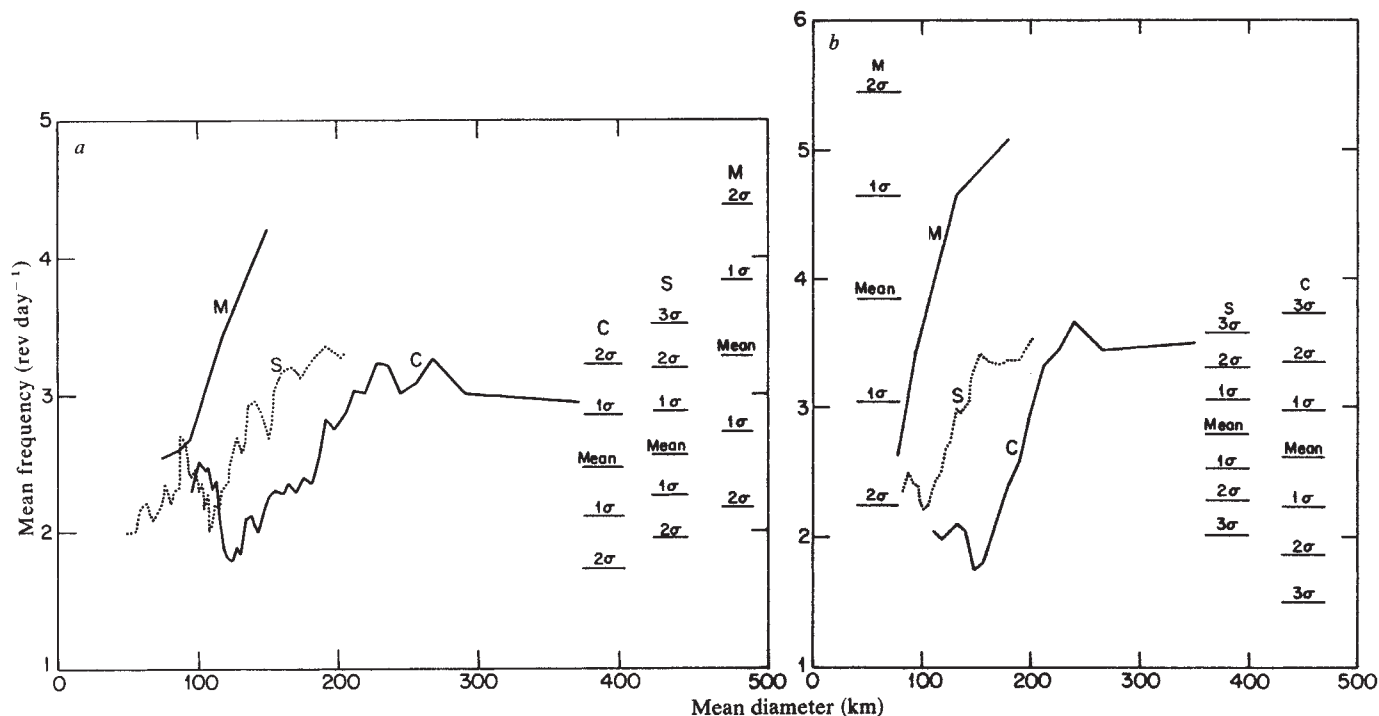


Fig. 3 A plot of the mean rotational frequency against the mean diameter for M, S and C asteroids listed by Tedesco and Zappalá⁴ and shown in Fig. 1, obtained by applying a running box method to the data which were sorted in order of increasing diameter. In *a* all data of quality ≥ 1 were included whereas in *b* only data of quality ≥ 2 were used. For the S and C plots the running box contained 10 asteroids. For the M asteroids the running box contained five asteroids in *a* and three asteroids in *b*. In all cases the box was stepped through the data one asteroid at a time.

The curves for each subset, but particularly those for S- and C-type asteroids, which are, of course, the best determined, are strikingly similar and contain several interesting features. Not all of these features are of strong statistical significance, but the fact that they are common to both the S and the C curves persuades us that they are probably real.

Each curve contains a near-linear portion for which we can write

$$\omega = mD + \omega_0 \quad (4)$$

Values of the slope m and of the intercept ω_0 for each near-linear portion are given in Table 1. All the values of m , but particularly those derived from the better quality data, are very similar. In both the S and the C curves shown in Fig. 3*b* there is a suggestion of a plateau at mean frequency $\sim 3.4 \text{ rev day}^{-1}$ (period $\sim 7 \text{ h}$). There are also significant discontinuities at small diameters. These are particularly evident in Fig. 3*a* which contains more data on asteroids with small diameters. The discontinuity on the C curve in Fig. 3*a* occurs at mean diameter of $\sim 125 \text{ km}$ at which point there is a 2σ increase in mean rotational frequency. A similar increase occurs on the S curve at mean diameter $\sim 105 \text{ km}$. We consider that the observed increases in mean rotational frequency could be due to fragmentation of asteroids on collision and that the discontinuities may divide primordial asteroids from their collisional products.

The most important feature of Fig. 3, however, is that the curves for M-, S- and C-type asteroids are separate. If asteroids of any one diameter are considered, then, on average, M asteroids rotate faster than S asteroids which in turn rotate faster than C asteroids. As we have proved that rotational frequency does depend on diameter and because the M-, S- and C-type asteroids in our chosen population have different mean diameters, it follows that a simple comparison of the mean frequencies of the different subsets is of little value. We need a method which shows the dependence of rotational frequency on both diameter and type and the plots shown in Fig. 3 are ideal for this purpose.

We consider, but we cannot prove, that the discontinuities at small diameters and the plateaus shown in Fig. 3 are probably real. If this is the case, then the linear portions of the plots

probably have the greatest significance. Using Fig. 3*b* and the data describing the linear portions of the plots given in Table 1, we can see that at any one mean diameter S asteroids rotate faster than C asteroids by a factor of $1.48 \text{ rev day}^{-1}$. For the S and C asteroids this represents a difference in mean frequency of $\sim 2.6\sigma$. Tedesco and Zappalá⁵ found the same dependence on type for S and C asteroids but not the large statistical significance. However, they averaged their data over a very wide range of diameters and only compared the mean rotational frequencies of one group of asteroids from each subset. Our method allows us to examine the dependence of mean rotational frequency on mean diameter and on asteroid type in a single diagram and in such a systematic way that we can see that for all mean diameters S asteroids rotate faster than C asteroids. In fact, the separation of the curves is such that it makes better sense to state that if any one mean rotational frequency is considered, then, on average, C asteroids are significantly larger than S asteroids which in turn are significantly larger than M asteroids. (Note that the latter statement is not based on Figs 2 and 3, but on plots, not shown here, of mean rotational frequency against mean diameter derived from data ordered in rotational frequency rather than diameter.)

The running box method of averaging data has two advantages over a conventional histogram. (1) All points on a given curve are based on data for the same number of asteroids and therefore all points have equal weight. (2) The trend and structure in a given data set and some indication of their statistical significances can be displayed in a single diagram.

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Resonant absorption by water polymers in the atmosphere

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There is much evidence both from atmospheric¹⁻⁹ and laboratory^{10,11} measurements that water vapour can show absorption of millimetre waves in addition to that predicted by models based on monomers¹² and dimers¹³. Constituents other than water can be eliminated as the cause of the extra absorption which is characterized by having a structured spectrum, increasing more rapidly with decreasing temperature than is expected from equilibrium phenomena and in the atmosphere showing a highly variable strength which is not simply correlated with total water content. The steep temperature dependence suggests invoking non-equilibrium water species to provide an explanation and this will account for variability as well as giving new prospects for understanding the spectral structure. A non-equilibrium model has, however, to be reconciled with what is known about supersaturation. It also opens the question of the nature of the energy source which is maintaining the steady state and allowing the extra absorption to be observed. I suggest here that water polymers containing about 50 molecules can, by collective acoustic resonances and postulated transition dipole moments, explain the observed component of absorption of millimetre waves by the atmosphere in some conditions. The polymers are metastable and are almost certainly the cause of supersaturation in cloud chambers but in these their lifetime is much greater than in the atmosphere.

Some examples of the structured spectra observed in high saturation and stable conditions given by natural fogs are shown in Fig. 1b, but the variability of the phenomenon causes most atmospheric observations to have poorer reproducibility than is shown by these. This has led some authors^{14,15} to question the existence of anomalous absorption and to suggest that experimental artefacts could explain the effects. The data in Fig. 2 counter this by showing that measurements made by different experimental techniques used at different sites have an underlying common behaviour. They also show that this cannot be attributed to simple variations in the amount of water in the path. Low or zero values of anomalous absorption in some atmospheric conditions are to be expected and indeed were invoked to account for a few of the observations reported in ref. 5. The non-equilibrium nature of anomalous absorption also makes it difficult to interpret directly temperature dependence measurements specifically aimed at examining the role of equilibrium water dimers. There is, however, provocative evidence in finding high values of ~ 1 eV in the exponent of a Boltzmann factor describing results where the dimer binding energy would give ~ 0.2 eV. The high values are also supported by cloud chamber observations shown in Fig. 3 in which non-equilibrium conditions were deliberately created. The interpretation of these temperature exponent values will be discussed later.

To explain the spectral structure, the polymers are modelled as spherical liquid droplets which are assumed to show macroscopic surface tension behaviour. (Droplet is defined here as a liquid drop of such a size that an appreciable fraction of its molecules are on the surface. It may or may not contain a minority concentration of foreign atoms and may or may not be charged.) They will then have predictable collective acoustic modes with eigenfrequencies determined by droplet radius but if these are to be effective in electromagnetic absorption the major assumption must be made that the oscillations are accompanied by transition electric dipole moments. No values for

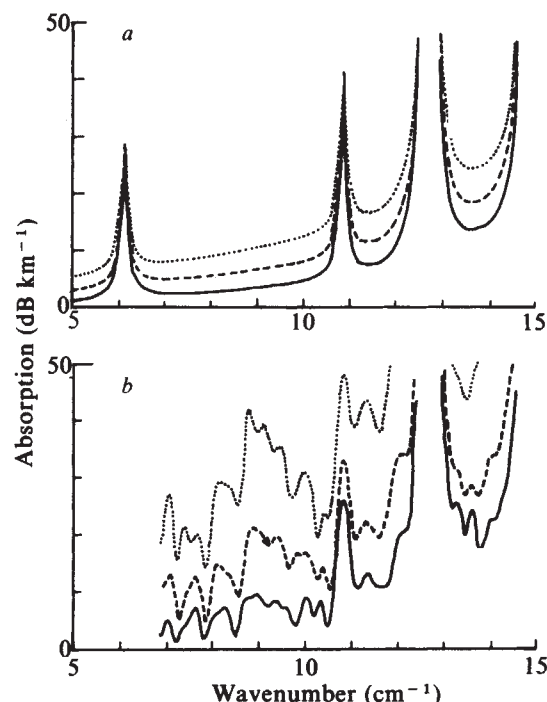


Fig. 1 *a*, Predicted spectra for fogs comprising molecular absorption and particle loss computed from standard models: corresponding to visibilities of 50 ($\cdots$), 100 ($---$) and 150 ($---$) m. *b*, Spectra observed in the atmosphere corresponding to the models. The standard deviation in the measured spectra is 3 dB km^{-1} . In such fogs the liquid drops are all small compared with the wavelengths and show Rayleigh region behaviour in which attenuation varies monotonically. Neither this component nor equilibrium dimers can account for the observed additional structure between the monomer lines.

these are known but arguments for their existence can be given from considering the molecular structure of droplets of appropriate size. A molecular model for the polymers is also necessary to account for the complexity of the observed spectra. Classical macroscopic arguments applied to measurements of supersaturation are, however, sufficient to show that droplets in the required size range exist in cloud chambers and there seems to be no reason that droplets with the same size distribution should not be found in the atmosphere with a much reduced lifetime. This could be as small as 10^{-10} s and droplets could still be effective in millimetre wave absorption. Their existence in the atmosphere is supported by the measured temperature dependence of absorption giving high values of exponents.

The formulation of the theory uses results due to Kelvin-Gibbs (equation (1)), Frenkel¹⁶ (equation (2)) and Landau-Lifshitz¹⁷ (equation (3)) relating the quantities S = supersaturation ratio; v_m = molecular volume in liquid phase (cm^3); σ_R = radius corrected surface tension (erg cm^{-2}); R = droplet radius (cm); R_c = droplet radius at critical supersaturation (cm); G = the droplet free energy at critical supersaturation radius R_c (eV); ρ = liquid density (g cm^{-3}); ω = acoustic capillary wave angular frequency in a sphere of radius R (Hz); l takes integral values 1, 2, ..., n with $l=2$ the lowest value for the droplet to depart from sphericity; $\omega = 2\pi c\bar{\nu}$ where $\bar{\nu}$ is in cm^{-1} for electromagnetic waves as justified; c = speed of light (cm s^{-1}). The three relationships are plotted in Fig. 4.

$$\log_e S = 2\sigma_R v_m / R_c kT \quad (1)$$

$$G = \sigma_R R_c^2 4\pi/3 \quad (2)$$

$$\omega = \sigma_R l(l-1)(l+2)/\rho R^3 \quad (3)$$

The values of surface tension have been corrected for dependence on R by the expression proposed by Tolman¹⁸ and though controversial its inclusion does not affect the arguments in any

critical way. The capillary waves are surface modes which for droplets will become the dominant acoustic eigenfrequencies. Further, as they describe motion of the liquid interface with free space and if accompanied by changes in dipole moment, the angular acoustic frequency can be related to electromagnetic oscillations using the free-space value for the speed of light.

If the curves of Fig. 4 are to be the basis of the kind of spectral structure shown in Fig. 1b, evidence must be given that some special significance can be attached to specific values of R . This comes in part from PVT observations¹⁹ in cloud chambers showing that if ions are not excluded a critical supersaturation ratio of about 4 cannot be exceeded but that at the critical value a long-lived state can be produced. Fig. 4a,b show that the corresponding specific droplet radius and free energy are $\sim 7 \times 10^{-8}$ cm and ~ 0.8 eV respectively.

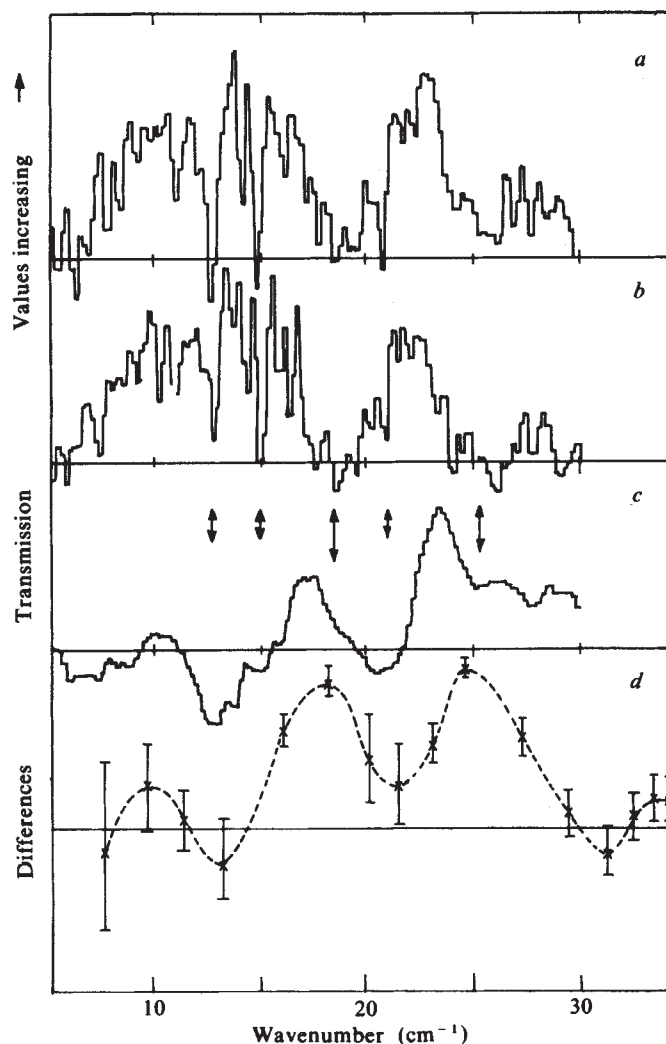


Fig. 2 a, b, Individual spectra of atmospheric emission measured successively within 30 min at Mount Evans, Colorado (see ref. 4). They represent relatively stable daylight conditions and show consistency in predicted water monomer and oxygen features but considerable variation at other wavenumbers. The difference between the two spectra shown by the smoothed curve of c has wavenumber maxima which do not coincide with the water lines indicated by the arrows and has both positive and negative values. It cannot therefore be attributed to varying amounts of water monomer. Curve d (derived from ref. 22) comes from a solar transmission spectrum observed at Gornergrat, Switzerland, with differences from prediction obtained by calibration with a black body. Again d has both signs and the resemblance to c which suggests that the cause of the differences is an atmospheric phenomenon common to the two sites and observable by different experimental methods.

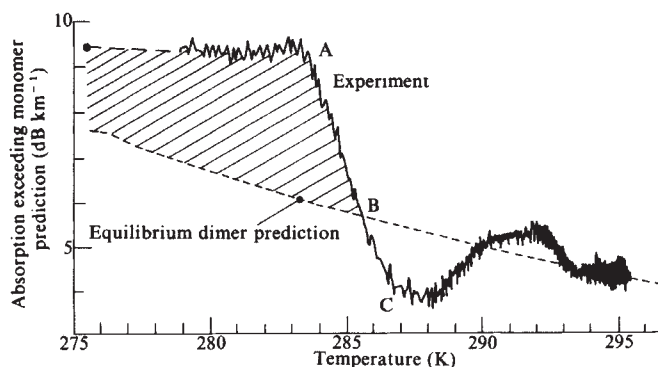


Fig. 3 Results on the temperature dependence of absorption of radiation in a band extending from 7 to 30 cm^{-1} by a mixture of water vapour and argon. The gas had been expanded adiabatically to a supersaturation ratio of 2.5 in a cloud chamber and the solid line represents the observed changes in absorption on its relaxing to room temperature. This behaviour differs considerably from a model shown by the dotted line which is based on equilibrium dimers being the only addition to water monomers. The actual absorption is attributed to metastable droplets for which, using the assumptions in the text, a free energy of ~ 1.8 eV can be deduced by comparing the observed slope with the predicted slope at point B. This is based on dimer binding energy being 0.18 eV per molecule. The result is taken from unpublished work by W. B. Johnson *et al.* of Rutherford-Appleton Laboratory.

The latter represents an energy maximum which acts as a barrier to nucleation and maintains the unstable equilibrium. While there is a local minimum in droplet density at the critical radius there is also a local maximum in droplet lifetime which is an operationally important quantity in supersaturation though classical theory does not consider this. While it cannot be rigorously proven there is also reason to believe that the coincidence of long lifetime with the energy maximum is responsible for the measured temperature dependence of millimetre wave absorption giving characteristic energies of the order of 1 eV and thereby pointing to the existence of droplets in the atmosphere of about the same size. Although these energies came from equilibrium analyses it seems that the Arrhenius type plots^{11,20} used were indicative of reaction rates rather than energy levels but that these quantities are likely to be related.

While these arguments can provide a basis for a specific radius value and hence with Fig. 4c single peaks in the spectrum for each l value, the behaviour shown in Fig. 1b suggests that a more complex distribution of droplet free energy with size must be found. Classical macroscopic theory cannot provide this so results are given from a model which considers the effects of molecular behaviour within the droplets. The model is due to Plummer and Hale²¹ and their values of energy for droplets with discrete values of n between 40 and 57 are shown by crosses in Fig. 4b. These show large variations in droplet energy with radius or a kind of 'magic number' behaviour where Frenkel's relation is monotonic. Because droplet densities will vary exponentially with energy and it seems reasonable to assume that the variation in absorption cross-section with n will be much slower, the Plummer and Hale values can be translated into changes in absorption with wavenumber which can be compared with observation. The calculated absorption change in going from $n = 55$ to $n = 57$ which corresponds to a spectral interval $\Delta \bar{\nu}$ of $\sim 0.5 \text{ cm}^{-1}$ is $\sim 60 \text{ dB km}^{-1}$. The maximum change of observed absorption in the same wavenumber interval in the region of 8 cm^{-1} is $\sim 15 \text{ dB km}^{-1}$. This shows that inclusion of molecular binding conditions can to a first order account for the observed spectral structure if the existence of transition dipole moments associated with the capillary wave oscillations can be justified. Also, from considering the molecular structure of droplets with $n \sim 50$ these moments are seen to be plausible. Macroscopic arguments require that both the total energy of the droplet and its surface energy be minimized and any molecular model must respect these conditions.

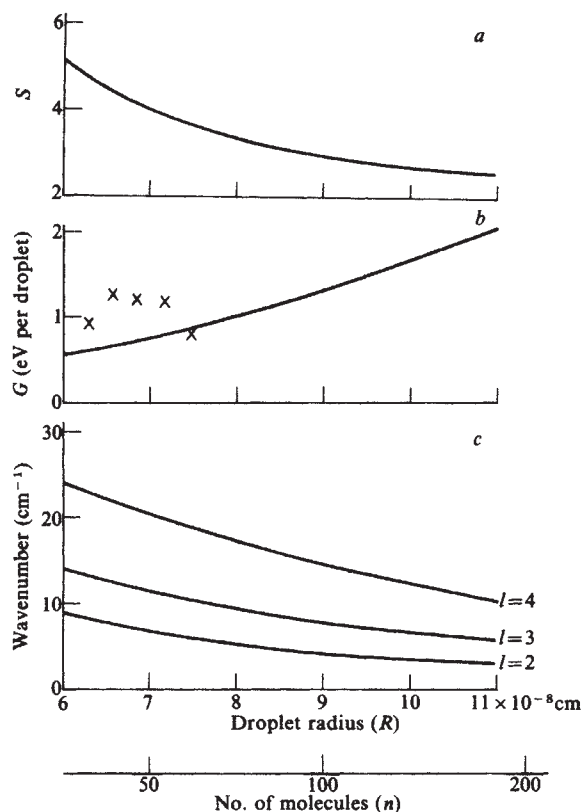


Fig. 4 Three quantities plotted against droplet radius R and the corresponding number of water molecules n . *a*, The supersaturation ratio S when the radius has the critical value R_c , given by equation (1) (Kelvin-Gibbs). *b*, The droplet free energy, G for the critical radius, R_c , given by equation (2) (Frenkel) with the crosses representing droplet energy for specific n values of 40, 45, 50, 55 and 57 given by the model of Plummer and Hale²¹. *c*, With the assumptions of the text gives the eigenwavenumbers $\bar{\nu}$ for electromagnetic absorption by droplets of radius R according to equation (3) (Landau-Lifshitz).

It seems unlikely that an additional constraint can then be imposed on a given size of droplet containing only about 50 polar water molecules with marked steric properties that it should show no change in dipole moment when constrained to make the changes in shape with l value required by equation (3). The condition for complete absence of structured absorption is even more stringent in that the transition dipole moments would have to be zero for all values of n in the range of interest for all capillary modes. These incidentally have a degeneracy value of $(2l+1)$.

The existence of metastable species raises the question of what are the likely sources of energy which are maintaining steady-state conditions. While no definite answer can be given as to whether the energy source is acoustic or electromagnetic in origin note that many of the observations in which the most marked anomalous spectral structure was observed were made in bright sunshine and that refs 22 and 23 are of particular interest in this connection. On the other hand in the laboratory measurements reported in ref. 11 no high energy photons were involved but the untuned resonator contained a source of acoustic power. In a purely acoustic experiment²⁰ high values of temperature exponent also suggest droplet formation.

The implications of the presence in the atmosphere of metastable absorbers and the need for energy sources to excite them greatly complicates the prediction of millimetre wave attenuation values. This complexity will almost certainly extend to other spectral regions²⁴ and will need to be considered in explaining observations. However, resonant absorption measurements could become a valuable new probe for the study of supersaturation.

An important feature of the present proposal is the introduction of collective acoustic oscillations and results on rough metal surfaces in the 4–12 cm^{-1} range from Raman scattering have been explained in the same way²⁵. A generalized treatment of the onset of collective modes has been given by Fröhlich²⁶ and this may contain the means of removing, at a later stage, the ambivalence of the present proposal which comes from the simultaneous use of classical and molecular model results. A great benefit of using the Landau-Lifshitz capillary wave formulation is that eigenfrequencies are given in terms of macroscopic parameters and thereby a useful starting point is obtained. I know of no other experimental results using the formulation but its use has been proposed to predict the behaviour of globular proteins²⁷ in scattering inelastic neutrons and the scattering of light by superfluid helium²⁸.

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Multi-armed vortices in an active chemical medium

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An excellent example of self-organization in nonequilibrium systems^{1,2} is the origination of rotating spiral vortices. These vortices have been observed in a wide range of active media—in the morphogenesis processes of social amoeba *Dictyostelium discoideum*^{3,4}, in cardiac muscle during some arrhythmias⁵ and in the Belousov-Zhabotinsky reaction^{6–9}. All these vortices are simple spirals. The rotating structures of a higher order of symmetry such as multiarmed vortices have not previously been observed experimentally. We have obtained two-, three- and four-armed rotating spiral vortices in an active chemical medium. These structures were appreciably stable and we observed their rotation for more than half an hour, which was in striking contrast to unstable multiple vortices in many other physical systems (such as superfluid ⁴He or superconductors^{10,11}).

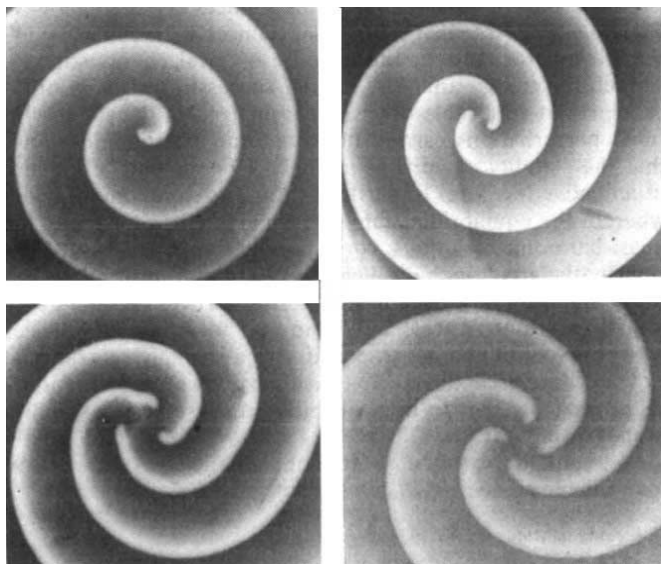


Fig. 1 One-, two-, three- and four-armed vortices in an active excitable chemical medium. A Petri dish 90 mm in diameter was filled with 4.5 ml of reagent similar to that proposed by Winfree: 0.3 M NaBrO₃; 0.4 M H₂SO₄; 0.1 M CH₂(COOH)₂ 0.85 M CHBr(COOH)₂; 0.045 M ferroin.

The multiarmed vortices were obtained as follows. Two, three or four waves were initiated close to each other by touching the solution with a silver pin. Wave-breaks were then induced by stirring a little of the reagent with the pin. One drop of 1 M KCl was placed close to the wave-breaks, and the waves began to circulate around it as though it were an obstacle (Cl⁻ interferes with the oxidation-reduction chemistry¹²). After one or two rotations the drop was removed by a strip of filter paper, whereupon a multiarmed vortex arose. The number of its arms equalled the number of waves preceding its appearance. The

idea of this procedure was to begin with a regime which would be *a priori* stable, and then change to the regime we were studying.

To increase the accuracy of measurements and to avoid the influence of the small amount of Cl⁻ that remained in the reaction mixture, the vortex was displaced outside the domain occupied previously by the drop of KCl solution. This was achieved by passing through the solution a train of concentration waves with a frequency exceeding that of the vortex. Thus, the vortex could be shifted without displacement of the liquid¹³.

The vortices obtained are shown in Fig. 1. Far away from the core, the multiarmed vortices form equidistant rigidly rotating spirals in accordance with the well known solution (2) of the reaction-diffusion equation. But in the core region the wave behaviour has proved to be unexpectedly complex: there was no simple rotation; instead, waves were intermittently connected as shown in Fig. 2. The geometry and the kinematics of the waves in the vortex core would not fit the modern theoretical concept of vortex rotation (see equation (2)).

The periods of waves (T) sent into the medium by multiarmed vortices were found to increase with the number of arms: $T_1 = 28 \pm 1$ s, $T_2 = 29 \pm 1$ s, $T_3 = 31 \pm 1$ s, $T_4 = 34 \pm 2$ s (the mean from 7–15 experiments $\pm$ s.d.).

All multiarmed rotating structures exist as long as the chemical reaction runs (>30 min). However, we also observed the decay of vortices. Figure 3 shows the disintegration of a two-armed vortex into two one-armed vortices. The disintegration was usually observed 50–60 min after mixing the reagents. Apparently, the disintegration was due to the change in active medium parameters with time. The two-armed vortices almost always decayed after 60 min of reaction life.

The decay of a three-armed vortex was observed in only one of seven experiments. However, in none of the experiments was the decay of a four-armed vortex evident. At the same time, all types of vortices were perfectly stable to external perturbations. The increase in temperature by 15 °C gave rise to a 2–2.5-fold decrease in the wavelength and the period, but

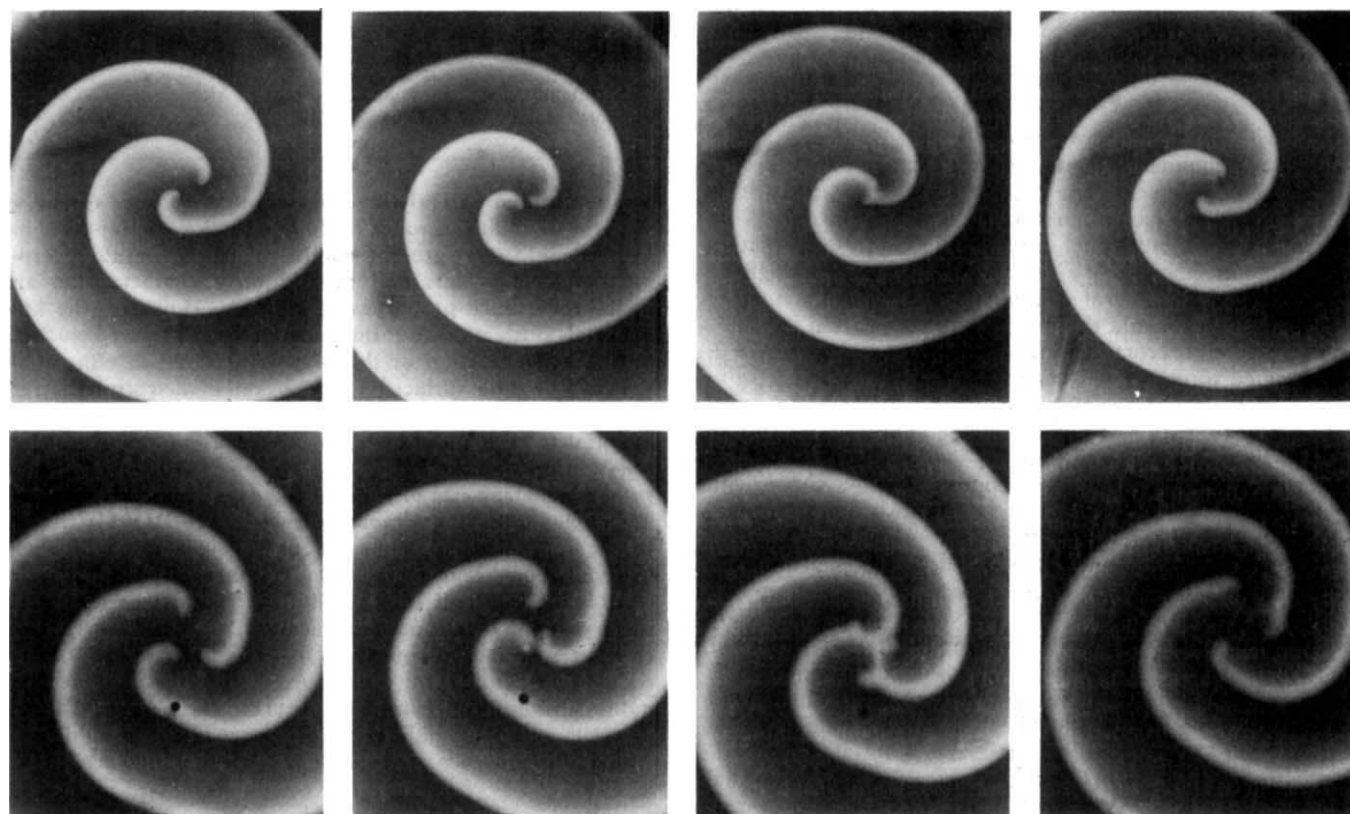


Fig. 2 Wave movement in the core of two- and three-armed vortices. Time interval is 15 s. Other conditions as in Fig. 1.

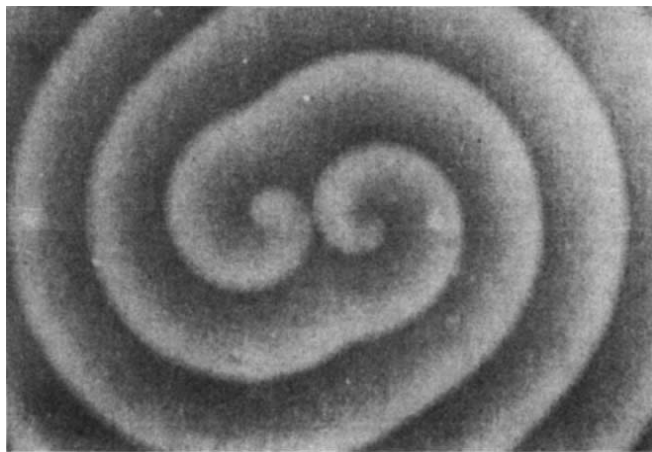


Fig. 3 Two closely spaced one-armed vortices formed from a two-armed vortex. The photograph was taken 58 min after mixing the reagents.

no signs of disintegration of multiarmed vortices were seen. In experiments where a multiarmed vortex was shifted by high-frequency concentrational waves, the structure of both the core and the outer region of the vortex was drastically changed, but in each experiment the vortex regenerated on switching off the external source of high-frequency waves.

Although similar structures to the multiarmed rotating vortices can be seen on some photographs of the aggregation of *D. discoideum*, these are not rotating waves. They are motionless and represent the path along which the cells are moving, whereas the form of the rotating concentration wave of cyclic AMP is a single one-armed spiral.

Active media are governed by the parabolic partial differential equation (the so-called reaction-diffusion equation):

$$\frac{\partial \mathbf{u}}{\partial t} = D \Delta \mathbf{u} + f(\mathbf{u}) \quad (1)$$

where $\mathbf{u}$ is the concentration vector; Δ , the laplacian; D , the diagonal matrix of diffusion coefficients. The vortex-like solutions of this equation of the form

$$\mathbf{u} = F(N\theta - \omega t, r) \quad (2)$$

are known¹⁰ where r and θ are the polar coordinates, and N is the integer number or the topological charge of a vortex^{10,11}.

This solution describes the rigid rotation of a vortex with a constant angular velocity ω in a two-dimensional medium. Note that in a three-dimensional medium the centres of rotation form a thread—the axis of a vortex—which may be straight or curved^{9,10}. The topological charge N characterizes the number of arms in a vortex and the direction of its rotation ($N = \pm 1$, a one-armed vortex rotating clockwise or anti-clockwise; $N = 2$, a two-armed vortex; $N = 0$, concentric waves).

The topological charge of the system of vortices is conserved during their formation, disintegration and annihilation both in physical systems and in active media—vortices emerge only in pairs with topological charges of opposite sign—a vortex with topological charge 2 may disintegrate into two simple vortices with topological charges 1, and so on.

Vortices with a topological charge $N > 1$ have been analysed with regard to the theory of superconductors, superfluid ⁴He and modern field theories^{11,14}. As the vortex energy is directly proportional to N^2 , vortices with a topological charge $N > 1$ are unstable in the physical systems considered. Similar results have been obtained by the theory of active media consisting of the van der Pol oscillators. It has been shown that unidirectional vortices repulse each other, which results in instability of vortices with $N > 1$ (ref. 10). These findings were the basis of the widespread opinion that the multiarmed rotating vortices cannot exist in real active systems because they must decay into simple ones as fast as formed.

How could we observe longlasting vortices with a topological charge $N > 1$? Note that energy is not conserved in active media, the waves propagate at the expense of local energy sources. Therefore, energy analysis, though successful in many other cases, is not applicable here. Linear analysis of the stability to small perturbations carried out after our experiments shows that the stability of vortices strongly depends on the kinetics of an active medium. In particular, for two-component media wherein the equilibrium state of each element is unstable, the quasi-harmonic solutions corresponding to vortices with a sufficiently large topological charge N are also unstable. But if the equilibrium state becomes stable, the vortices become stable for all values of N (ref. 15). Note that we obtained vortices with topological charges 2, 3 and 4 in a strongly relaxational excitable (not oscillating) active medium. However, no theoretical approach seems to be available to analyse vortex stability in that case.

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Surface microlayer phenolic enrichments indicate sea surface slicks

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The realization by early investigators that oil films spread on surfaces of bodies of water could mimic slick conditions^{1–3} has evolved into the present model of slicks resulting from monomolecular films that modify wave characteristics and hence reflectance properties of surfaces. However, the lipid materials postulated to form monomolecular layers constitute only a small fraction of the organic material in either bulk seawater or at the surface^{4–7}, may not retain their strong surface activity in the presence of natural seawater dissolved organic material (DOM)⁸, and have not been found in any consistent relationship with slicks^{7,9–11}. The apparent association of slicks with macroalgae has also been noted^{12–15}, but there has not been a demonstration of macroalgae-derived DOM in slicks. I show here that enrichments of UV-absorbing phenolic materials, consistent components of surface microlayers¹⁶, offer reliable indication of slick conditions. I then suggest that: (1) slicks result not from monomolecular films, but from viscosity changes in surface microlayers caused by more soluble organic components; and (2) the occurrence of slicks therefore depends not on absolute concentrations of surface-active materials, but on relative viscosity differences. Evidence is also given demonstrating the presence of macroalgae-derived phenolic materials in slicks.

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Surface microlayers were collected from Maine estuarine and coastal waters and from the North Atlantic ocean by the glass plate method^{17,18}. Microlayer samples and appropriate reference bulkwater samples were each filtered through pre-combusted, rinsed glass fibre filters. The filtrates were assayed for phenolic materials using UV absorbance at 280 nm in 10 cm quartz cells and a modification of the Folin-Ciocalteu test¹⁹, and for DOC by persulphate oxidation and IR detection^{20,21}. Microlayer enrichments were calculated as the ratio between the concentration of a substance in microlayer and in bulkwater samples. Particular attention was given to surface conditions at the time of sampling. The following classification system was used: (1) certified slicks—visually obvious slicks with at least some distinct boundaries; (2) slick influenced samples—conditions in which, although distinct boundaries were absent, the surface had some combination of stable bubbles, particulate debris, or viscous appearance during sampling which was characteristic of certified slicks; (3) mixed samples—samples in which some dips of the plate were in areas designated as certified slick or slick influenced surfaces and some were in clean areas;

Table 1 Slick and clean enrichments for phenolic materials and DOC

Type	<i>n</i>	Phenolic enrichment		<i>n</i>	DOC enrichment	
		$\bar{x}$	Range*		$\bar{x}$	Range
Certified	30	2.49	1.52–6.12† (0.192–1.04)	8	1.39	0.79–1.79† (2.92–11.87)
	31	3.33	1.52–28.54 (0.192–2.34)	9	1.78	0.79–4.92 (2.92–18.10)
				4‡	2.39	1.65–3.57 (1.27–4.43)
Influenced	24	1.69	1.41–2.47 (0.137–0.421)	6	1.74	0.89–4.68 (3.65–11.19)
Mixed	13	2.00	1.48–3.06 (0.172–0.555)	2	0.81	0.58–1.03 (2.15–9.46)
Clean	97	1.27	0.99–1.88 (0.125–0.405)	41	1.13	0.54–1.54 (1.82–12.32)
				5‡	2.82	1.84–3.73 (1.91–3.92)

* Range is range of enrichments; ranges of absolute values, in units of absorbance at 280 nm and mg l^{-1} DOC, respectively, are in parentheses.

† Data set excludes extreme values from a dense slick so as not to bias comparisons with other categories. Extreme value is included in next data set.

‡ Data included from ref. 14.

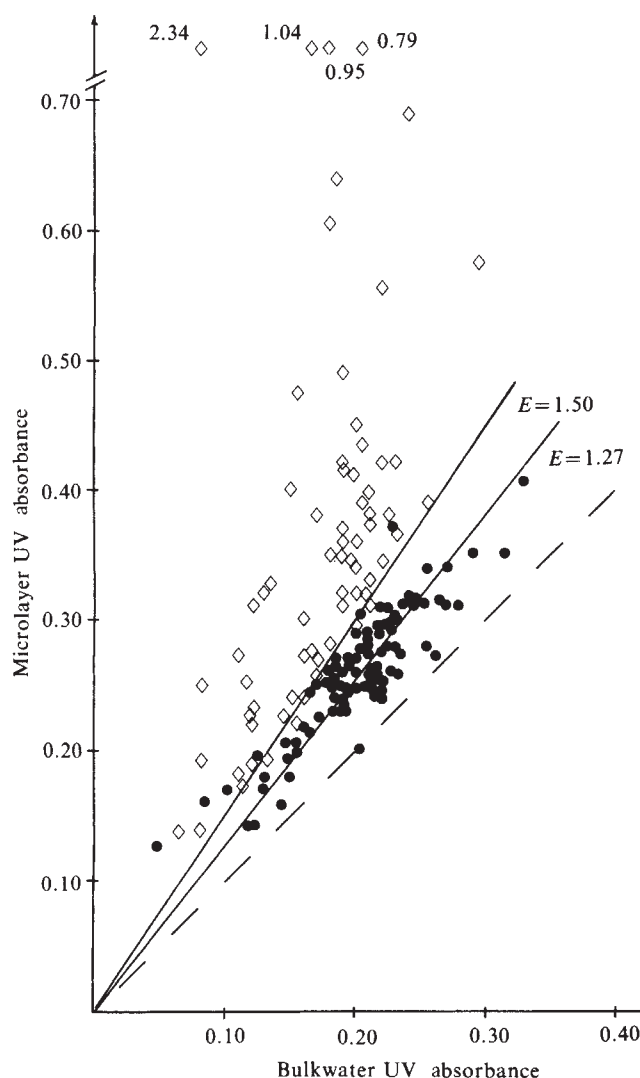


Fig. 1 Microlayer phenolic content versus bulkwater phenolic content, as measured by absorbance at 280 nm. Data were collected May 1978 to August 1981 from estuarine, Gulf of Maine, and North Atlantic (39° N, 66° W) waters. Lowest line indicates equal absorbance in microlayer and bulkwater samples. Line with slope of 1.27 ($r^2=0.99$) denotes mean phenolic enrichment of clean microlayer samples. Line showing enrichment of 1.50 represents boundary between clean and slicked microlayers. ●, Clean surfaces; ◇, slicked surfaces.

and (4) clean surfaces—no slicks, no visible accumulations of debris, stable bubbles rare or absent.

The slick data are summarized in Table 1, with clean surface data included for comparison. Only the phenolic materials showed a consistent relationship to slick conditions. All three slick classifications had mean phenolic enrichments greater than did clean surfaces. The mean phenolic enrichment in certified slick samples was significantly greater ($P < 0.05$) than the mean enrichment in slick influenced samples, with the mixed sample enrichments intermediate to and not significantly different from either the certified or influenced samples. In contrast to the phenolic materials, slick DOC enrichments were not significantly different ($P > 0.05$) from clean enrichments. Although the highest DOC concentration was in a dense slick, high DOC concentrations were not consistently related to the occurrence of slicks. DOC data from Pacific coastal and oceanic waters were similarly non-representative of the presence of slicks¹⁴. DOC as measured undoubtedly includes, in addition to relatively insoluble materials contributing to surface enrichments, varying quantities of more soluble materials. Therefore, it is not surprising that neither enrichments nor concentrations of DOC were reliable indicators of slick conditions. Phenolic materials are, however, preferentially enriched in microlayers relative to DOC¹⁶, and they represent the first fraction of DOC which consistently indicate slick conditions.

The slicks were distinguished by enrichment of phenolic materials rather than by concentration (Fig. 1). Slicks were evident throughout the UV absorbance ranges of both subsurface and microlayer samples, indicating that no minimum amount of phenolic materials was necessary for slicks to appear. Instead, the line representing an enrichment ratio of 1.50 clearly distinguished slicked from clean surfaces. This observation, that slicks were indicated by relative (enrichment) rather than absolute (concentration) measures, independent of the type of material measured, is inconsistent with the model of wave-damping by an incompressible film. In that model, wave-damping requires some critical concentration of surface-active molecules, independent of bulkwater concentration, above which slicks should ideally always be evident and below which no slicks should occur. The phenolic evidence indicates a relative relationship—slicks becoming evident when the UV absorbance in the microlayer reaches a ratio of 1.50 relative to the UV absorbance in bulkwater, compared with an average clean surface ratio of 1.27. I propose that the relative nature of the slicks observed here, indicated by phenolic materials, is due to relative differences in microlayer viscosity.

In organic-free seawater, damping of capillary waves at a clean interface is related to the viscosity of the seawater²². Organic materials in natural seawater will alter the viscosity of both the bulk seawater and, if they accumulate near the surface,

of the surface microlayer where the viscosity changes may be enhanced by interfacial effects. When the amount of organic material in a microlayer increases the viscosity relative to bulk seawater and to adjacent but less enriched microlayers so that wave characteristics are altered, slicks will become evident. Slicks thus become a function of the viscosity in surface microlayers rather than of the compressibility of a monomolecular film, and the appearance of slicks depends on relative viscosity differences between slicked and clean microlayers, both relative to bulkwater.

One implication of a relative viscosity hypothesis is that at a constant bulkwater viscosity, a microlayer viscosity gradient should exist between fully clean and fully slicked surfaces. In confirmation of this, the slick influenced samples did have a mean phenolic enrichment intermediate to clean and certified slick samples. In addition, *in situ* measurements of 'film pressures', which are generally performed without definitive demonstration of the presence of surfactant monolayers, probably represent an interaction not between the spreading oils

and a monomolecular film but between the spreading oils and the natural microlayer organic materials. From this viewpoint, there is some evidence of a gradient from clean surfaces to weak and then sharply defined slicks²³ which supports the suggestion of the relative nature of slicks.

A second implication is that as the concentration of DOM in both clean and slicked microlayers increases in response to increased bulkwater DOM, the viscosity will also increase. Although the appearance of a slick in one area will depend on viscosity differences relative to clean surfaces in that area, both the slick and clean surfaces should have greater viscosities and different capillary wave characteristics relative to slick and clean surfaces in an area of lower bulkwater DOM. For example, in the low DOM North Atlantic samples, visual distinction between slicks and clean surfaces was less certain than in coastal waters, perhaps because of lower surface DOM concentrations and lower viscosities. It is possible that such differences between areas of different DOM, for both clean and slicked surfaces, might be detectable and quantifiable by remote sensing instruments, and alternatively, might affect measurements by such instruments.

It remains then to consider the UV-absorbing phenolic materials as constituents of slicks, and their sources. The IR evidence of Baier²⁴ suggesting highly hydroxylated components in natural slicks could apply to phenolic materials. A polymeric nature has been suggested for terrestrial and macroalgal materials showing phenolic characteristics^{25,26} and the phenolic materials might be expected to interact with amino acids, carbohydrates, or lipid materials²⁷⁻³⁰ to form complexes similar to hypothesized microlayer compounds^{7,24,30}. The complex chemistry of the phenolic materials in seawater precludes assigning DOC values to their UV absorbance (refs 31, 32 and D.J.C., L. M. Mayer and M. L. Brann, unpublished data). There are, however, indications that microlayer materials and substances extracted from fucoid macroalgae can increase the solution viscosity of seawater³³, although it remains difficult to extrapolate that information to viscosities in microlayers.

It has been shown that in low UV absorbance, low phenolic content offshore and oceanic waters, it is difficult in both bulkwater and in microlayers to distinguish terrestrial and macroalgal sources for the phenolic materials¹⁶. In slicks, however, where the amounts of UV-absorbing materials can be large, there was definite evidence that macroalgae-derived phenolic materials contributed to slick DOM (Fig. 2). In Maine coastal waters, macroalgae have a seasonal exudation pattern³² which, perhaps accompanied by seasonal changes in reactivity of the exuded phenolics (D.J.C., L. M. Mayer and M. L. Brann, unpublished data), may contribute to the seasonal occurrence patterns of slicks¹⁸.

The above observations do not preclude other compounds, such as lipids, as components of slicks, or negate physical slick formation processes, such as convergences. However, whatever the formation processes and regardless of additional components, enrichments of UV-absorbing phenolic materials provided consistent indications of slick conditions in all coastal waters sampled. It is possible that phenolic materials may be ubiquitous components of clean and slicked surface microlayers and might serve as indicators of slicks in other areas, especially coastal areas, of the oceans.

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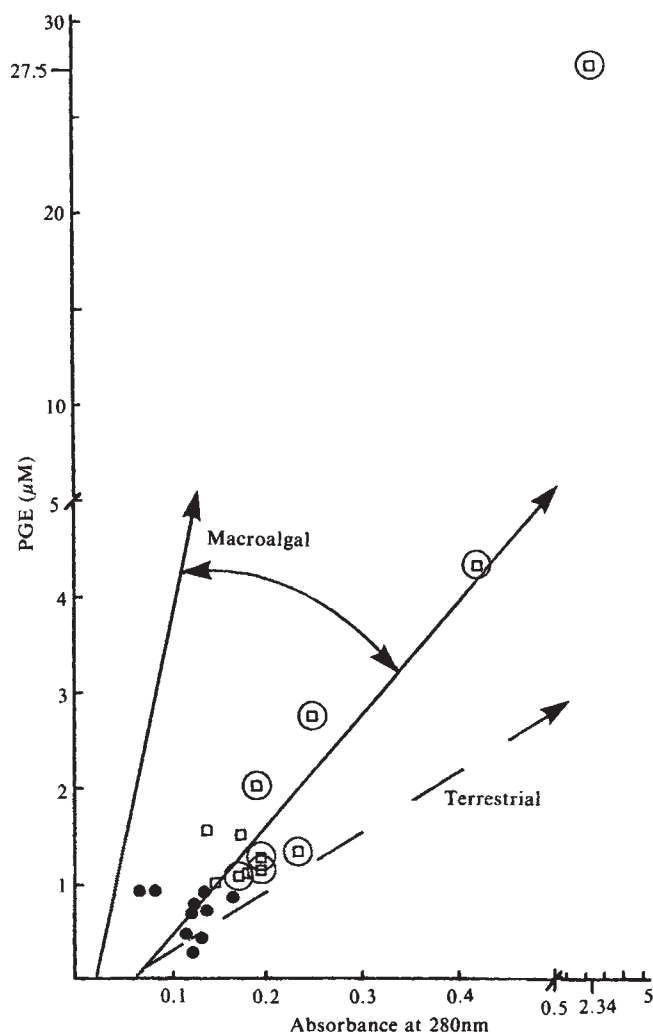


Fig. 2 Absorbance at 280 nm plotted versus phenolic reactivity expressed as micromoles of phloroglucinol equivalent (PGE). Dashed line indicates ratios typical of terrestrially-derived phenolic materials, while solid lines outline ratios of macroalgal-derived materials¹⁶. Sources of bulkwater (●), clean microlayer (□) and slicked microlayer (⊙) materials with absorbances ≤ 0.20 cannot generally be distinguished. However, in some slicks with higher absorbances and reactivities, ratios indicated macroalgae-derived material. Samples collected October 1979, August 1980, and August 1981 in coastal waters of the Gulf of Maine, 0–30 km offshore. Salinity ranges: 29.5–33‰. Scales change at 0.5 absorbance and at 5 μM PGE.

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Lead 210 and moss-increment dating of two Finnish *Sphagnum* hummocks

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Peat materials are economically and ecologically valuable and so their dating is important. The lead isotope ^{210}Pb (22.26 ± 0.22 yr) has had great value in dating recent deposits^{1–8} of the past 150 yr. But the varying conditions in different water reservoirs, and the possibility that the mathematical models used in ^{210}Pb -dating will give different results, call for additional chronological data.¹⁴ C-dating has major difficulties in this respect^{8–10} and dating with ^{137}Cs and ^{239}Pu is not reliable^{10–13}. Previously ^{210}Pb -dating has been tried on only seven peat cores from ombrotrophic mosses: four from Denmark^{14,15} and three from England and Ireland¹⁰. We present here a comparison of ^{210}Pb results with moss-increment dates of selected peat material from Finland.

Two *Sphagnum fuscum* hummock cores from two Finnish peatlands, Karpansuo bog, Kuhmoinen ($61^\circ 35' \text{N}$, $25^\circ 20' \text{E}$), core 1, and Kunonniemensuo bog, Kite ($62^\circ 05' \text{N}$, $30^\circ 10' \text{E}$), core F9, were used in this study. These cores had been previously dated by the moss-increment method^{11,16}. Both sampling sites are small, ombrotrophic, raised bogs with scattered small pines (*Pinus silvestris* L.) and derive their water supply exclusively from precipitation. The average water table in both hummocks is ~ 30 – 35 cm below the peat surface. The average peat depth at the sites is ~ 4 m. Most of the peat moss layers had grown in ombrotrophic conditions (according to pollen analysis these layers are younger than 6,000 yr BP), while the bottom layers of 50 cm or so were formed in minerotrophic conditions. The hummocks from which the cores were taken are several metres in diameter. They consist of a very loose *S. fuscum* carpet with dwarf shrubs such as *Empetrum nigrum*. Some *Polytrichum strictum* was found at both sites and the annual increments of this species served as a dating tool. The cores were taken with the 12-cm diameter steel corer and cut into sections, 2.5–5.0 cm thick. The peat material was slightly decomposed white moss (*Sphagnum*)^{11,16}.

The shape and microstructure of the individual mosses were used to identify the annual increments. These could be distinguished in *Sphagnum* by means of cyclic pigmentation¹⁷, branching pattern¹⁸ and changes in growth direction caused by pressure from the snow cover¹¹. About 15–20 stems of *Sphagnum* mosses and/or *P. strictum* vertically penetrating the peat sections were measured to determine their actual lengths after straightening. From these stems, the annual increments (which include 1 yr of growth) were identified¹⁹ and their lengths measured. In the case of very short increments a stereomicroscope ($\times 150$) was also used to observe the changes in the stem pigmentation. The average number of years within each section was calculated by dividing the mean stem length with the mean increment length, so the values used represent an age interval in moss-increment years. Because the averages of the age intervals were not statistically weighted, no statistical distribution of the moss-increment years in the age intervals were given^{11,16}.

Materials in the same peat samples were analysed for their ^{210}Pb contents at the Institute of Physics, Uppsala, Sweden and the Technical Research Centre of Finland, Espoo, Finland. The samples from the Karpansuo bog, core 1, contained only peat moss (*S. fuscum*) materials and all the visible high (vascular) plant remains were excluded. The samples from Kunonniemensuo bog, core F9, comprised the entire peat material. However, the percentages of the vascular plants were low in all but the deeper subsamples.

The 'total' ^{210}Pb was measured in the two laboratories by determining the granddaughter product ^{210}Po by means of the isotope dilution. The ^{208}Po was used as an internal yield tracer in both laboratories. These tracers were calibrated against different standards. The chemical separation of polonium samples in the two laboratories is described elsewhere^{6,20,21}. The samples were counted by α spectrometry using silicon surface barrier detectors. The long-term stability of these detectors was considered²². The radon emanation was used in Uppsala to determine ^{226}Ra (ref. 23). The ^{226}Ra was separated from the sediment residues, with the help of Ba-carrier, and kept in closed bottles for a month. A nitrogen gas was then used to flush the ^{222}Rn to an ionization chamber that was carefully examined for impurity and adsorption effects^{6,20} in particular.

The total ^{210}Pb of the peat samples comprises ^{210}Pb of atmospheric origin; unsupported ^{210}Pb and an additional *in situ* part which is produced from ^{226}Ra ; and supported ^{210}Pb . The supported ^{210}Pb may not always be in secular equilibrium with its precursor ^{226}Ra . Supported ^{210}Pb was estimated at Espoo only from the total ^{210}Pb of samples older than 200 moss-increment years. The unsupported ^{210}Pb for such samples should be $< 2\%$. In Uppsala the ^{226}Ra ($t_{1/2} = 1,620$ yr) was also used to evaluate the supported ^{210}Pb . The ^{226}Ra values of five samples, three of them being taken from one core, are in good agreement and the average value is 0.12 ± 0.04 pCi g^{-1} . This value agreed with the total ^{210}Pb values in the deeper samples showing 200–500 moss-increment years; the averages of the two laboratories are 0.07 ± 0.01 pCi g^{-1} and 0.19 ± 0.03 pCi g^{-1} for core F9 and core 1 respectively.

^{210}Po ($t_{1/2} = 138$ days) is assumed to be in equilibrium with ^{210}Pb ($t_{1/2} = 22.26 \pm 0.22$ yr) in all but the surface slice (^{210}Po reaches secular equilibrium with ^{210}Pb in about 2 yr). This assumption is valid only in ideal cases of closed systems in which neither the ^{210}Pb nor the ^{210}Po is subject to any kind of translocation in the time interval of interest. This assumption was confirmed in a sediment core²⁴. Lewis²⁵ estimated a mean residence time for metals similar to ^{210}Pb in organic-rich soils to be about 2,000 yr. Mosses of ages younger than 700 yr showed that 95–99% of the total lead is either bound or sorbed¹⁴ to the peat materials. Jacobsen¹⁵ found that selected suboceanic ombrotrophic peats from Denmark have a very high capacity for retaining metals and could be used to give good records on atmospheric deposition, especially for lead 'petrol lead'. Pakarinen and Tolonen²⁶ claimed that mobility of lead might

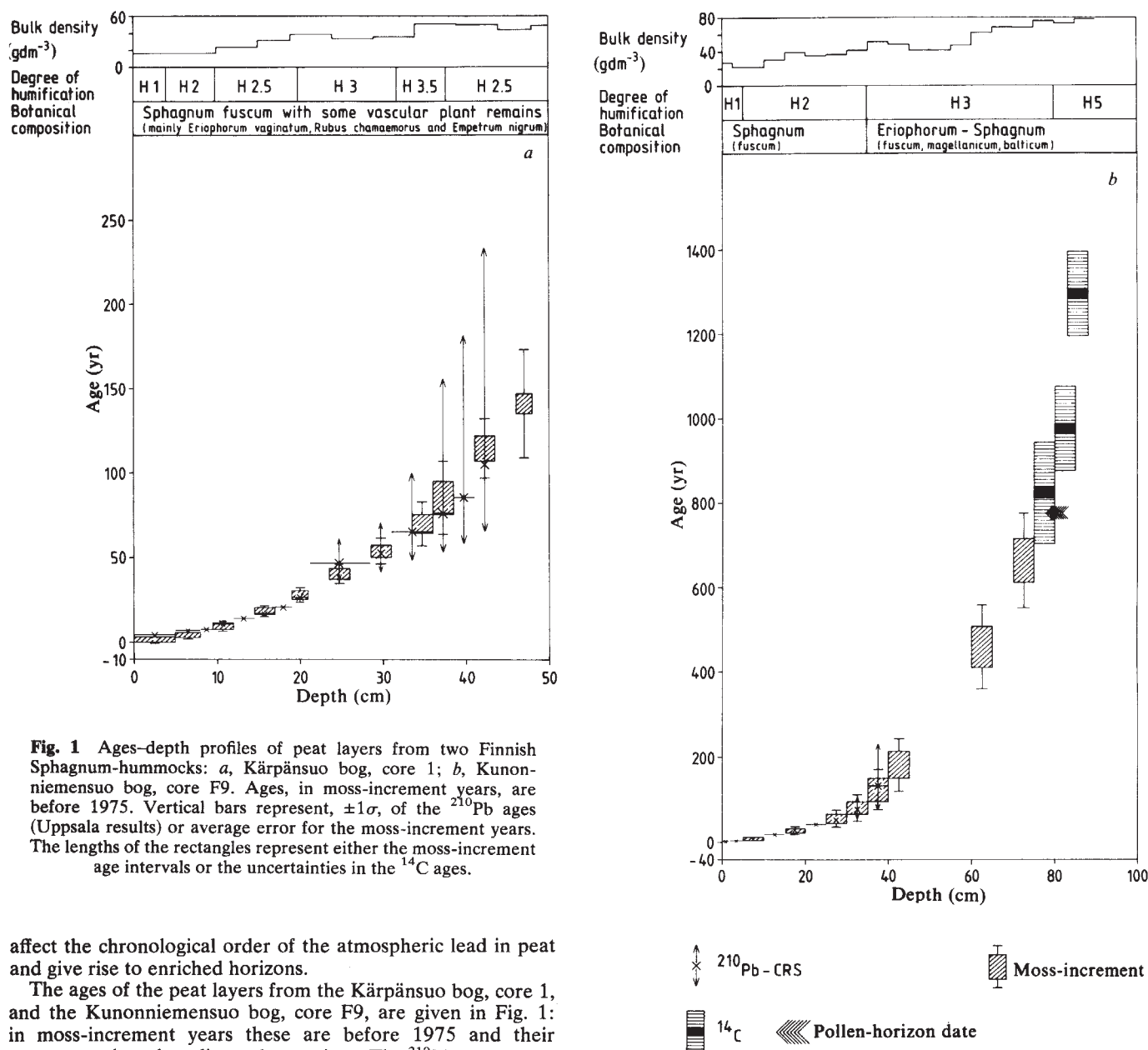


Fig. 1 Ages-depth profiles of peat layers from two Finnish Sphagnum-hummocks: *a*, Kärpäsuo bog, core 1; *b*, Kunonniemensuo bog, core F9. Ages, in moss-increment years, are before 1975. Vertical bars represent, $\pm 1\sigma$, of the ^{210}Pb ages (Uppsala results) or average error for the moss-increment years. The lengths of the rectangles represent either the moss-increment age intervals or the uncertainties in the ^{14}C ages.

affect the chronological order of the atmospheric lead in peat and give rise to enriched horizons.

The ages of the peat layers from the Kärpäsuo bog, core 1, and the Kunonniemensuo bog, core F9, are given in Fig. 1: in moss-increment years these are before 1975 and their errors were based on direct observations. The ^{210}Pb -concentrations (per unit dry weight) which were measured at Uppsala and Espoo, using different chemical extractions and independently calibrated tracers, allow tracing of systematic errors in the laboratories and lend credibility to the evaluation of the ^{210}Pb models. The uncertainties in the ^{210}Pb values included not only the statistical uncertainties in the ^{210}Po activity measurements but also those due to the ^{208}Po tracers, the corrections of the polonium peaks for the silicon-detector instabilities (performed only in Uppsala) and the supported ^{210}Pb activity. For ^{226}Ra , the uncertainties included the counting statistics, the background of the ionization chamber and the efficiency of the filling system as determined by a standard sample. The ages in ^{210}Pb yr were calculated using the constant initial concentration (CIC) and the constant rate of supply (CRS) models^{27,28}. The ^{210}Pb concentrations of the past 20–30 moss-increment years (the upper 15–20 cm) were normalized for their radioactive decay and a σ -weighted average was calculated for each core and laboratory. These averages served as initial concentrations for the studied cores (CIC model), and smoothed down horizontal variations of bulk densities and growth rates which took place in the surface peat layers. Such variations may cause the ^{210}Pb concentrations to differ from place to place even if the atmospheric supply rate of the ^{210}Pb was constant. These values, as calculated with aid of the least

squares method, were: for core 1, Kärpäsuo bog $5.50 \pm 0.22 \text{ pCi g}^{-1}$ for Uppsala and $4.70 \pm 0.35 \text{ pCi g}^{-1}$ for Espoo; for core F9, Kunonniemensuo bog, the Uppsala value was $5.43 \pm 0.27 \text{ pCi g}^{-1}$ and for Espoo $5.99 \pm 0.44 \text{ pCi g}^{-1}$. For the CRS model the integrated activities over the past 150 yr were: for the Kärpäsuo core, $33.3 \pm 3.0 \text{ nCi m}^{-2}$ and $44.8 \pm 3.6 \text{ nCi m}^{-2}$ for Uppsala and Espoo respectively; the corresponding values were $49.2 \pm 3.3 \text{ nCi m}^{-2}$ and $47.3 \pm 2.9 \text{ nCi m}^{-2}$ for the Kunonniemensuo core.

Figure 1 shows the age-depth relation for both cores. The ^{210}Pb ages, as calculated using the CRS model, show good agreement with the moss-increment dates. The radiocarbon dates for some deeper layers from core F9, Kunonniemensuo bog, are also given: Helsinki-1140, 75–80 cm, $800 \pm 120 \text{ yr BP}$; Helsinki-1053, 80–85 cm, $950 \pm 100 \text{ yr BP}$; and Helsinki-1141, 83–88 cm, $1,270 \pm 100 \text{ yr BP}$. The dates were derived using Libby's half life (5,570 yr). The samples were used without further physical pretreatment and without the NaOH-insoluble fraction being dated. Unfortunately no $\delta^{13}\text{C}$ values are available to correct for the isotopic fractionation but if the suggested values for peat materials²⁹ which range from -19% to -35% are used, an age correction by 95 to -145 yr could be expected.

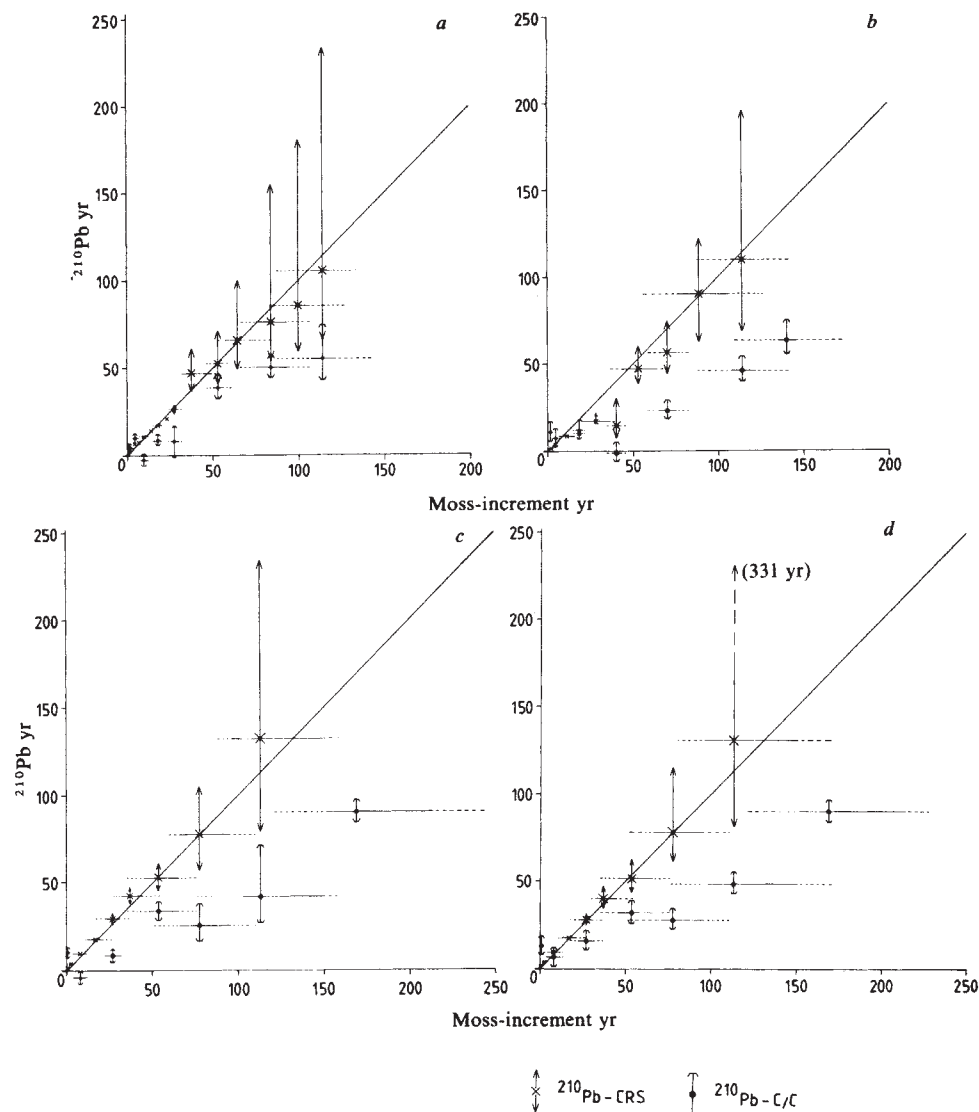


Fig. 2 A comparison between the ^{210}Pb years as calculated using the CIC and CRS models and the moss-increment years of the Kärpäsuo bog, core 1: *a*, Uppsala results; *b*, Espoo results and the Kunonniemensuo bog, core F9; *c*, Uppsala results; *d*, Espoo results. Vertical bars represent standard deviation, $\pm 1\sigma$, of the ^{210}Pb ages; the horizontal bars give the age intervals of the peat layers with their average errors in broken bars.

Thus, the ^{14}C dates show good agreement with the moss-increment dating and the distinct onset of rye cultivation at 80 cm, which according to pollen studies by Vuorinen on an adjacent varved lake (Hinnisenlampi), occurred ~AD 1200. The bulk density of the top moss layers is lower than the underlying layers because of either or both compaction and decomposition of the peat materials. For both cores the CIC model underestimates age compared with the moss-increment method (Fig. 2) which suggest higher initial concentrations of the 'unsupported' ^{210}Pb in the deeper moss layers. This coincides with the calculations made on the annually accumulated dry matter which showed lesser net accumulation for the earlier peat layers^{11,16}. The CRS model shows a good agreement with the moss-increment method (Fig. 2). All the dates agreed within $\pm 1\sigma$ except for three dates from Espoo (the Kärpäsuo core) which deviated by -2σ . Note that the CRS model gives rise to higher statistical uncertainties in the final ages. As one moss-increment year represents a growing period which corresponds quantitatively to one calendar year, it seems likely that the atmospheric rate of the ^{210}Pb supply during the past 150 yr was constant, the annual peat layers of the cores effectively accumulated the available ^{210}Pb during their growth and the peat material behaved as a 'closed' system.

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Seasonality and mean annual sea surface temperatures from isotopic and sclerochronological records

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Seasonality is one of the most dramatic climatic variables affecting the physical and biological properties of the surface of oceans. Attempts to deduce the seasonal temperature contrast of past oceans using the fossil record have led investigators to explore the carbonate growth patterns in certain marine invertebrate organisms. In many cases these growth patterns represent annual growth increments analogous to annual rings in trees¹. Just as tree rings contain a record of terrestrial climates², systematic variations in the physical or chemical properties of annual growth increments in the skeletons of marine organisms record the environmental or climatic properties of the marine realm. Several investigations have called attention to the use of stable isotope profiles in growth increments of mollusc shells to determine the seasonal water temperature changes³⁻⁷ and seasonal timing of coastal upwelling⁸. Mollusc shells therefore have the potential of revealing much information about past environmental changes⁹, particularly along temperate continental shelves where our knowledge of the palaeoceanographic history is very limited. We have now used coordinated isotopic and sclerochronological (growth increment) studies to compare the geochemical history of shell growth in *Spisula solidissima* with the physical oceanographic history of the Middle Atlantic Bight and determine whether the isotopic records of *S. solidissima* from different water depths on the continental shelf reflected the seasonally different thermal regimes at those depths. We chose *S. solidissima* because it is widely distributed along temperate continental shelves, its shell growth and life history are well understood^{9,10}, and the genus has a fairly wide distribution in Plio-Pleistocene fossil deposits. Jones^{11,12} has already established through a detailed sclerochronological analysis of *S. solidissima* that a 10–20 yr time series of mean sea surface temperature trends can be resolved from the record of annual shell growth. A strong negative correlation exists between growth increment size and yearly mean water temperature¹¹.

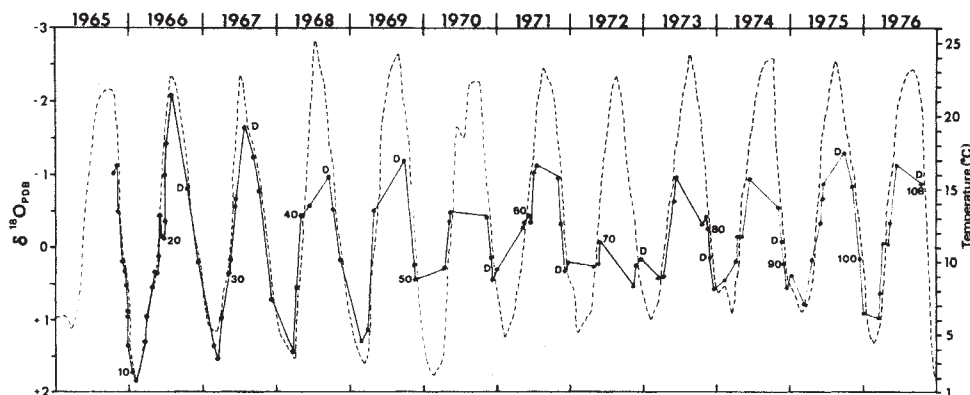
Live specimens of *S. solidissima* were collected from two localities off coastal New Jersey in the Middle Atlantic Bight, from water depths of 10 and 45 m. Growth increment analyses were performed whereby yearly changes in shell growth height were corrected for ontogenetic growth trends^{11,12} by dividing the measured shell height by the expected growth predicted using the Van Bertalanffy curve as described by Caddy and Billard¹³. This treatment yields a standardized index that can be compared among individuals of any size (age)¹¹. Between 7 and 23 isotopic samples of aragonite powder were secured from each annual growth band by microdrilling or filing of the outer shell layer. The inner layer was avoided to eliminate potential isotopic effects due to shell dissolution and reprecipitation. Annual dark growth increments in the outer layer provide reference points to differentiate successive years' of growth and to prevent sample overlap¹¹. Stable isotopic analyses were performed according to established procedures¹⁴ (all isotopic data are given in δ notation as the ‰ enrichment or depletion relative

to the PDB carbonate standard¹⁵). A total of 150 separate isotopic analyses were made for an 11-yr period (1965–76) from the inshore 10 m specimen (INS no. 1), and for the calendar year 1966 in two offshore specimens from 45 m water depth (OFF nos 3, 5). The stable isotopic data sets for the calendar year 1966 were compared with surface and bottom temperature and salinity data available for the collection area^{11,16,17}. The exact year of growth could be determined if the collection date was known and the sclerochronological analysis of each shell^{9,11} performed.

The 11-yr isotopic time series from the 10-m site exhibits a regular periodicity and confirms that the $\delta^{18}\text{O}$ of the outer shell layer of INS no. 1 monitors the record of changing seasonal water temperature but not in a linear fashion (Fig. 1). The yearly isotopic amplitude ($\Delta^{18}\text{O}$) decreases steadily from 1965 to 1972 and increases again from 1972 to 1976. The $\Delta^{18}\text{O}$ values have a maximum range of 3.8‰ during the first year of growth (1966). A straightforward interpretation of the isotopic range in terms of seasonal contrast would suggest wrongly that a steady decrease in seasonal contrast occurred from 1965 to 1972, with the seasonal amplitude increasing in years 1973–77. This interpretation is not supported by historical oceanographic data for the Middle Atlantic Bight^{16,17}. Shell growth in *S. solidissima* is apparently inhibited, but not totally stopped, during years with unusually warm mean sea surface temperatures (MSST) ($>12.5^\circ\text{C}$), and the observed yearly $\Delta^{18}\text{O}$ is reduced. During 1972, for example, when MSST was 13°C , the $\Delta^{18}\text{O}$ values would suggest a seasonal contrast of $<5^\circ\text{C}$ and unusually warm winter minimum and cold summer maximum temperatures (Fig. 1). Sclerochronological analysis of INS no. 1 revealed that the addition of shell height was maximum during the first year of growth and decreased almost exponentially until the eighth year of growth when a slight increase occurred again. Jones¹¹ had previously demonstrated that the size of annual growth increments in populations of *S. solidissima* are highly correlated with yearly mean sea surface temperatures. Shell growth conditions are apparently most favourable during years which have generally cooler water temperatures (MSST $<12.5^\circ\text{C}$). A plot of the yearly isotopic amplitude ($\Delta^{18}\text{O}$) versus a standardized growth index and mean sea surface temperatures (Fig. 2) reveals a similar relationship. The yearly isotopic range ($\Delta^{18}\text{O}$) is negatively correlated ($r = -0.77$) with MSST and shows a strong positive correlation with the growth index ($r = 0.79$). $\Delta^{18}\text{O}$ values are therefore large and closer to the annual isotopic amplitude expected from the seasonal temperature contrast when MSST conditions are favourable to growth (Fig. 1). Periods of abnormally low growth thus best explain the diminished $\Delta^{18}\text{O}$ values. The $\Delta^{18}\text{O}$ record is, therefore, best interpreted as a function of MSST, and not as a measure of the annual temperature contrast or seasonality except possibly in optimum years of growth. The significant correlations in Fig. 2 suggest that the $\Delta^{18}\text{O}$ values may be used to estimate the MSST of the Middle Atlantic Bight to within better than 0.5°C .

It is desirable, therefore, that growth increment analyses be done concomitantly with detailed stable isotopic analyses of molluscs to interpret seasonality information from mollusc growth records properly. Even though the $\delta^{18}\text{O}$ at the outer shell margin of a particular mollusc may seem to reflect the approximate season that a specimen is collected, the isotopic amplitude cannot be strictly interpreted in terms of seasonality. The $\Delta^{18}\text{O}$ in particular species must be interpreted in the context of the growth history of the particular mollusc as was emphasized as early as 1953 (ref. 3). In the use of *S. solidissima*, we are fortunate that calibration of the $\Delta^{18}\text{O}$, growth increments and seasonality can be made before investigation of fossil specimens. Once this calibration is made, it should be possible to decipher quantitatively important properties of continental shelf waters using isotopic and sclerochronological studies of fossil specimens. Although it is not possible to use *S. solidissima* to obtain a time series of seasonality, the mollusc may have the potential for recording the seasonal temperature amplitude on

Fig. 1 Oxygen isotope signal (solid line) for 11 yr of growth in a specimen of *Spisula solidissima* (INS no. 1) collected alive from a depth of 10 m on the Mid-Atlantic Bight off New Jersey. The dashed line corresponds to the monthly mean sea surface temperature for 1965–76 at the collection site. Isotopic data within a given year can be plotted knowing the growth rate of the specimen and the approximate position of the 'dark' growth increment which is laid down during the late summer–early autumn months^{11,12,18}.



the continental shelf. A test of this can be seen in Fig. 3 where the $\delta^{18}\text{O}$ signal for the year 1966 from INS no. 1 is plotted relative to the record of sea surface temperature (SST). The $\delta^{18}\text{O}$ data yield two types of information: (1) that the magnitude of the seasonal temperature contrast was accurately tracked; (2) that shell growth was continuous but varied systematically throughout the year in the following manner. Growth rates were most rapid during late spring–early summer (March–June).

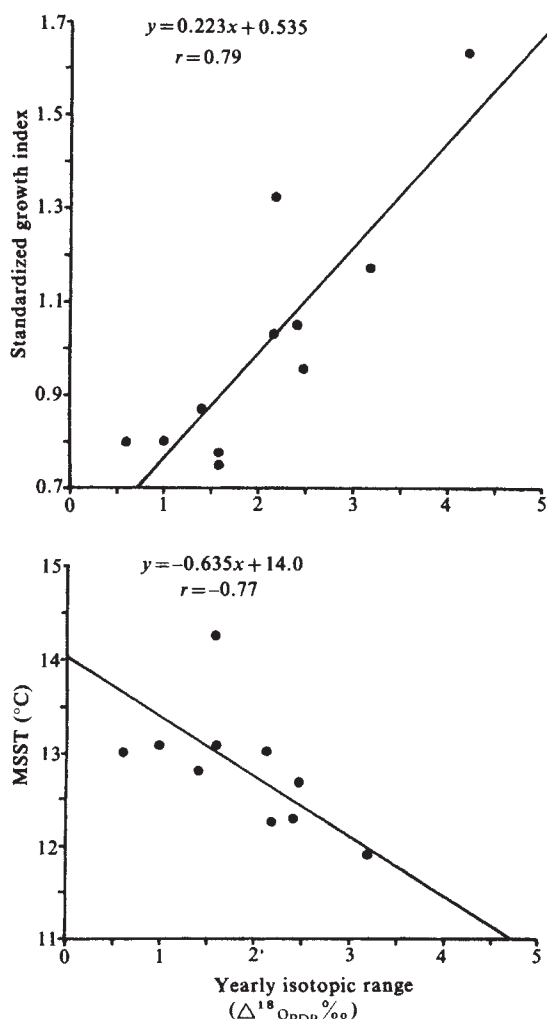


Fig. 2 The relationships of the yearly isotopic range ($\Delta^{18}\text{O} \text{ ‰}$) with the standardized growth index^{11,12} and yearly mean sea surface temperatures (MSST) for 11 yr of growth in *S. solidissima* (INS no. 1). The best fit lines from linear regression analysis are also shown. The standardized growth index is computed by dividing the measured shell height by the expected growth using the Van Bertalanffy growth curve^{11–13}.

In July, growth slowed but decreased to a minimum during the SST maxima in August through October, when spawning and deposition of the characteristic dark increment occurred. Shell growth then increased again as SST cooled in October through December. This confirms the annual cycle in shell growth that has been quantitatively defined through biweekly observations of living *S. solidissima* populations from this region of the continental shelf^{12,18}.

To determine whether these results could be used to differentiate the thermal structure on the continental shelf off New Jersey, the detailed $\delta^{18}\text{O}$ record for the year 1966 in INS no. 1 (10 m) was compared with that of two specimens collected live from 45 m water depth (OFF nos 3, 5) (Fig. 3). During January to April–May, the 10 m and 45 m specimens have identical $\delta^{18}\text{O}$ signals. From May to September, the 10 m and 45 m records clearly diverge as the surface waters continue to warm and the temperature gradient increases to a maximum in August. As the surface and 45 m water temperatures begin to converge in October to January, so do the $\delta^{18}\text{O}$ values of the inshore and offshore specimens. These trends lead us to believe that the isotopic records of these specimens are accurately monitoring seasonal contrasts in the surface waters as well as the seasonal development and breakdown of the thermocline between the inner and middle shelf¹⁹. As fossil specimens of *S. solidissima* which grew at different depths may be differentiated on the basis of their shell morphology and growth curves¹², it may be possible to reconstruct the thermal stratification along the continental shelf from fossil shells.

A detailed sclerochronological and stable isotopic study of annual growth increments in *S. solidissima* collected live has enabled us to establish confidence limits about the use of mollusc isotopic data in palaeoseasonality studies. Sclerochronological analysis enables optimum years of shell growth to be identified. Although the annual isotopic signal ($\Delta^{18}\text{O}$) is qualitatively recorded in all years of growth, the magnitude of the $\Delta^{18}\text{O}$ in all years is not linearly related to the seasonal sea-surface temperatures. The first years of growth in *S. solidissima* seem to yield the best potential for quantitatively determining the seasonal contrast. It is possible, therefore, to use the isotopic data in *S. solidissima* to monitor successfully the development of the thermocline between the inner (10 m) and middle (45 m) continental shelf when the growth histories of the mollusc shells are understood. The carbon isotopic record of *S. solidissima* may also be utilized to decipher seasonal productivity and/or water mass stratification²⁰. Thus, calibration of the isotopic signals of molluscs like *Spisula* within a physical and biological framework may provide a powerful means to reconstruct the palaeo-oceanographic history of temperate continental shelves, especially during the Plio-Pleistocene for which numerous fossil deposits are available. Such valuable palaeoclimatic information has hitherto been unavailable from temperate continental shelf areas, and only possible where corals are present. Due to the thermal tolerances of mollusc species, however, caution may be required in palaeoseasonality studies of other mollusc species.

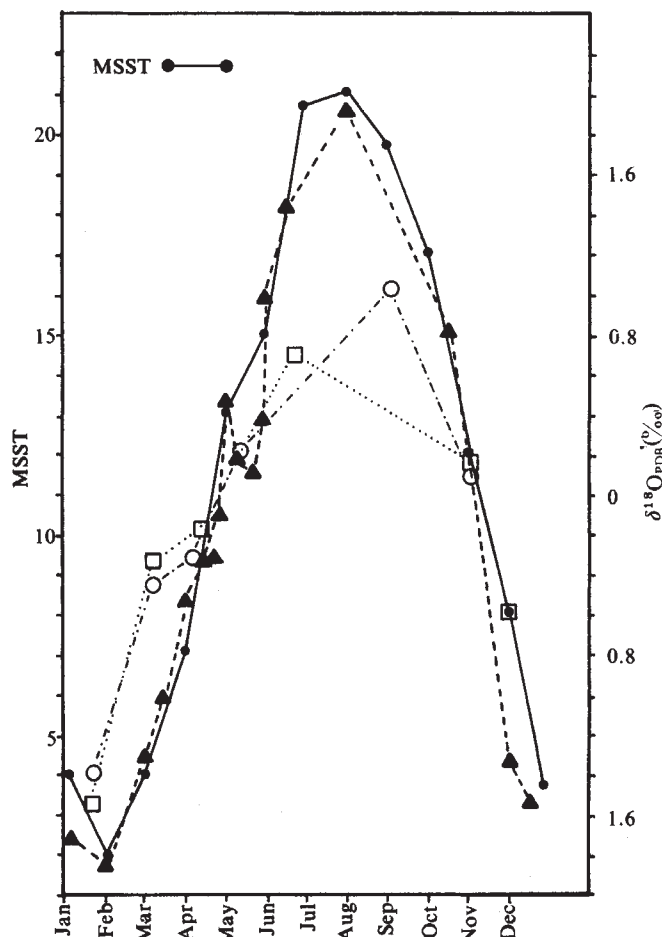


Fig. 3 A comparison of the seasonal change in monthly MSST for 1966 and the oxygen isotopic composition ($\delta^{18}\text{O}\text{‰}$) of the same year of growth in *Spisula* from water depths of 10 m (INS no. 1 ▲) and 45 m (OFF nos 3 ○; 5 □). The isotopic data are plotted knowing the growth rates of the individual specimens and the position of the 'dark' growth increment which is laid down during the late summer-early autumn months^{11,12,18}.

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Sedimentary record of rainfall variations in a sub-humid lake

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The physical, chemical and fossil characteristics of lake sediments are frequently used to study environmental processes and change within a lake, in the lake catchment and over a wider area. Analysis of sediment dry weight variations in a 1.3-m core from a lake, Dayat Afougach, in Morocco suggests that they record both seasonal and annual rainfall variations and the associated catchment erosion and lake sedimentation processes. If this is a common feature of certain lake sediments then these lakes may prove to be a source of information about changing precipitation patterns in areas where climatological records are not available, as well as providing information on changing sediment yields from their catchments.

Dayat Afougach (31°21' N, 5°07' W) is a 32 hectare eutrophic lake situated at an altitude of ~1,500 m in the Middle Atlas Mountains. The catchment area of the lake is roughly 1,400 ha and it is underlain by Mesozoic calcareous rocks¹. The mean depth of the lake is around 6 m and it has an outflow but no perennial inflow. The lake is in a semi-arid region², but it has a fairly high annual rainfall (mean for 1927-49) of 1,100 mm (ref. 1) due to its high altitude. The climate corresponds to the Mediterranean winter-wet summer-dry sub-humid climate type (see Fig. 4a). A basic limnological description of the lake has been made by Gayrals³.

An undisturbed sediment core⁴ was taken at the deepest part of the lake in the south-east from 11 m on the 25 September 1979. There was no visible stratification of the calcareous sediment. It was sectioned at 1 or 2 cm intervals and the sediment dry weight found after drying to 105 °C. The sediment was rendered soluble by HF-HClO₄ digestion⁵ and organic content estimated by loss on ignition⁶. Metal concentrations in the digests were determined by atomic absorption spectrophotometry, phosphorus colorimetrically⁷ and the precision of the results estimated by duplicates. The standard deviations for seven duplicates are 16 mg Ca per g, 3 mg Mg per g, 0.3 mg Na per g, 0.3 mg Fe per g, 0.06 mg P per g, 5 µg Mn per g, 2 µg Cu per g, 6 µg Zn per g, 15 µg Pb per g and loss

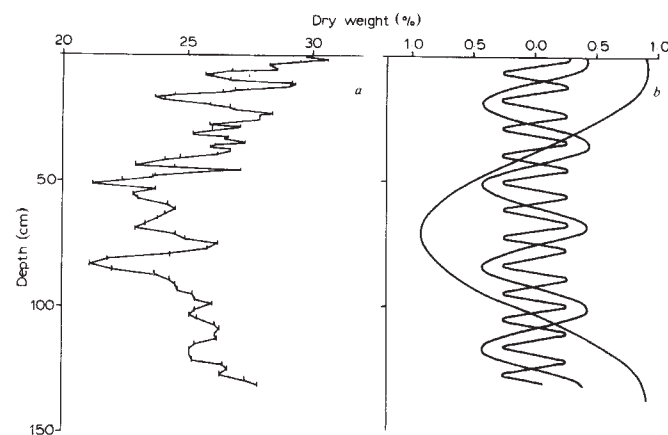


Fig. 1 a, The variation of sediment dry weight with depth in Dayat Afougach. b, The three Fourier harmonics, first, fourth and eleventh, which describe the dry weight behaviour in a. The scale indicates how much each harmonic causes the dry weight to depart from its mean value of 25.4%.

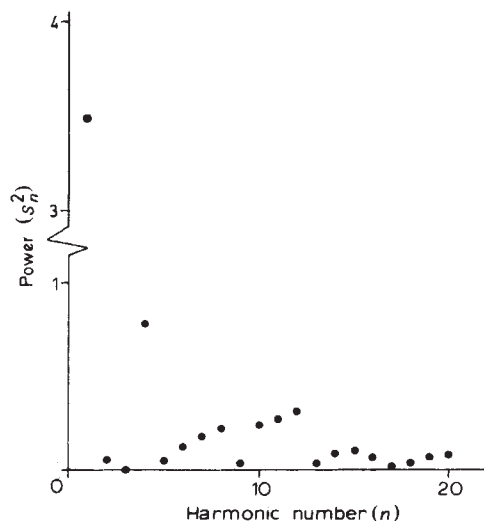


Fig. 2 The Fourier power spectrum of the dry weight results.

on ignition 1.3%. The dry weight results and annual rainfall totals from 1958 to 1979 for Ifrane, 23 km south-west of Afougach⁸, were subjected to Fourier analysis⁹.

The variation of sediment dry weight with depth (Fig. 1a) shows repetitive patterns and the Fourier power spectrum is shown in Fig. 2. The first harmonic accounts for 48.5% of the variance and has a wavelength of 131 cm, the length of the sequence. The sequence is too short to provide any evidence for a repeating unit of this wavelength. This harmonic only describes the general trend of increasing and decreasing values. An F-test shows the fourth harmonic accounts for a significant ($0.005 < P < 0.01$) amount of the variance (11.0%). It has a wavelength of 33 cm. The sixth, seventh and eighth harmonics, even taken as a group, are not significant ($0.05 < P < 0.10$). The tenth, eleventh and twelfth harmonics together make a significant contribution to the variance ($0.005 < P < 0.01$) and account for 11.9%. They have wavelengths of 13, 12 and 11 cm, respectively, and so with a wavelength around 12 cm account for the smallest scale repeating pattern in the dry weight results (Fig. 1a). Decomposed eleventh, fourth and first harmonics are shown in Fig. 1b. The contribution of the fourth harmonic is best seen when it is in phase with the eleventh harmonic at the pronounced dry weight minima around 15, 50, 83 and 120-cm depth. Two lines of evidence indicate that these dry weight patterns reflect rainfall variations.

The first concerns the smallest scale pattern, harmonics 10/11/12. This has a wavelength around 12 cm and is probably caused by an annual sedimentation cycle. The sediment accumulation rate would then be around 12 cm yr^{-1} . Three pieces of evidence support this interpretation. First, Wilson¹⁰ has shown that there is a strong relationship between monthly sediment yield and monthly rainfall in catchments with a Mediterranean type climate. Assuming that the sediment has a larger particle size during the periods of higher yield, then the variation in monthly rainfall over a year (see Fig. 4a) could cause the smallest scale dry weight pattern by controlling the grain size of the allogenic sedimentary inputs and hence the dry weight^{11,12}. During the wetter months, especially those following the dry season¹⁰, the allogenic inputs would be coarser than during the drier months and would produce the sediment layers of higher dry weight. Second, the relationships of organic content, sodium, magnesium and iron concentration to dry weight (Fig. 3) also indicate that the dry weight variations are due to particle size variations. Organic content and sodium and iron concentration are higher in clay-sized sediments¹³⁻¹⁵ and magnesium is frequently higher in the silt fraction^{15,16}, so the dry weight axis in Fig. 3 corresponds to particle size. None of the other elements determined (Ca, P, Mn, Cu, Zn, Pb) show

a significant relationship with dry weight. That the calcium concentration is constant suggests that the seasonal precipitation of calcium carbonate is not responsible for the patterns observed. Third, the implied accumulation rate of around 12 cm^{-1} is reasonable in this environmental setting. Around $1-22.5 \text{ cm yr}^{-1}$ have been found in reservoirs with varying catchment size in the drier areas of the USA^{17,18} and a rough sediment yield from the catchment of around 900 tonnes $\text{km}^{-2} \text{ yr}^{-1}$, calculated assuming the accumulation rate, a mean sediment wet density of 1.25 g cm^{-3} , no losses through the outflow and even sedimentation, is also reasonable in this environmental setting. Sediment yield is very variable but

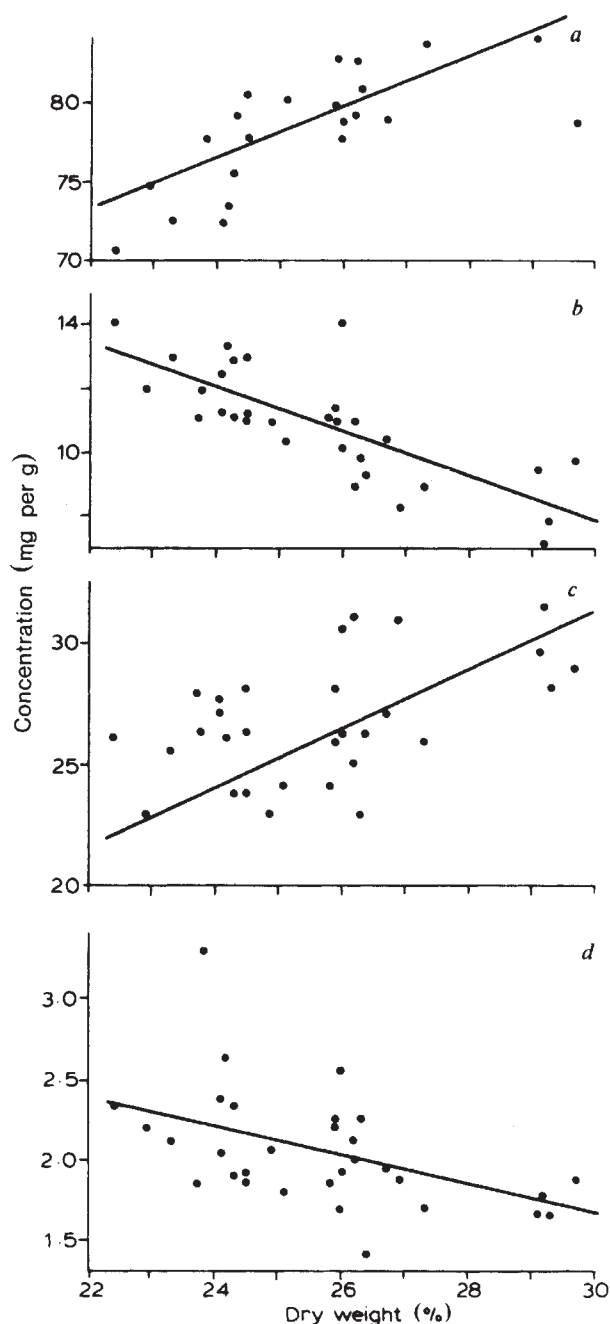


Fig. 3 The relationships between mineral content (a), iron (b), magnesium (c) and sodium (d) concentration and sediment dry weight in Dayat Afougach sediment. a: % mineral = $40.7 + 1.5 \text{ wt\%}$, $n = 23$, $r^2 = 0.38$, $0.001 < P < 0.01$. b: mg Fe per g = $28.8 - 0.698 \text{ wt\%}$, $n = 31$, $r^2 = 0.57$, $P < 0.001$. c: mg Mg per g = $9.9 + 0.659 \text{ wt\%}$, $n = 31$, $r^2 = 0.25$, $0.001 < P < 0.001$. d: mg Na per g = $4.4 - 0.091 \text{ wt\%}$, $n = 31$, $r^2 = 0.24$, $0.001 < P < 0.01$.

around 600 and 700 tonnes $\text{km}^{-2} \text{yr}^{-1}$ are suggested (ref. 19 and ref. 20 respectively) for this setting. The dry weight results in Fig. 1 show that, although the mean distance between peaks of the smallest scale pattern is around 12 cm, the distance varies from 8 to 18 cm. This behaviour appears to be accounted for by some of the harmonics on either side of 10/11/12, especially 7, 8 and 15, and may be due to annual variations in sediment yield from the catchment. Also, the dry weight–depth profile becomes damped and rounded below 100 cm. This may be due to compaction and to the 2-cm intervals used in sectioning the core at this depth. However, overall the three pieces of evidence suggest that the smallest scale repeating dry weight pattern reflects seasonal rainfall variations.

The second line of evidence suggests that the other two components of the dry weight variation reflect annual rainfall variations. Annual rainfall totals for Ifrane from 1958 to 1979 are shown in Fig. 4b and their Fourier power spectrum in Fig. 4c. The sequence is too short to provide reliable evidence that the third harmonic, with a wavelength around 7 yr, is a real environmental feature. This harmonic does, however, describe the decreasing rainfall in the early 1970s followed by a recovery, a pattern that was found over the whole of North Africa². Assuming the 12 cm yr^{-1} accumulation rate, then the low sedi-

ment dry weight values between 50 and 90 cm, described by the first harmonic, correspond to the years 1971–74. Thus the general trend of decreasing and increasing sediment dry weight (Fig. 1a and first harmonic in Fig. 1b) reflects the trend of decreasing and increasing annual rainfall at Ifrane in the 1970s (Fig. 4b). The seventh and eighth harmonics in the Ifrane rainfall results have wavelengths of 2.9 and 2.6 yr. Having a wavelength <3 yr this component is reflected in the dry weight results by the fourth harmonic, which, assuming a 12 cm yr^{-1} accumulation rate, corresponds to a wavelength of 2.8 yr. This, on average, just less than 3 yr rainfall cycle is probably part of the widespread quasi-biennial oscillation of many meteorological properties, including rainfall^{21,22}. The quasi-biennial oscillation has also been found in lake varves and tree rings²³. That the fourth and eleventh dry weight harmonics are in phase (Fig. 1b) also supports the suggestion that the fourth harmonic corresponds to year to year variations.

The above evidence, although indirect, does provide a coherent picture of sedimentation in Dayat Afougach greatly influenced by rainfall variations. Establishing the accumulation rate directly by sediment dating and particle size analysis is, nevertheless, desirable to increase the reliability of the interpretation. Unavoidable loss of the core prevented particle size analysis being carried out. The alternate sediment layers of different chemical constitution and particle size in Afougach may be termed seasonal rhythmites and these have been found in other lakes^{24,25}, including semi-arid ones¹⁷. The seasonal rainfall variation at Afougach seems to provide the periodic variation in allogenic sedimentary input. Although annual variations in the flood events in another lake were found to be recorded in the sediment dry weight profile¹⁸, whether the calcareous nature of the Afougach sediment is important in allowing rainfall variations to be recorded is not known at present. However, as the monthly sediment yield from catchments in areas with seasonal climates is dependent on rainfall¹⁰, sediment dry weight results may be a source of rainfall information in such areas, especially where there is little physical or biological mixing of the sediment.

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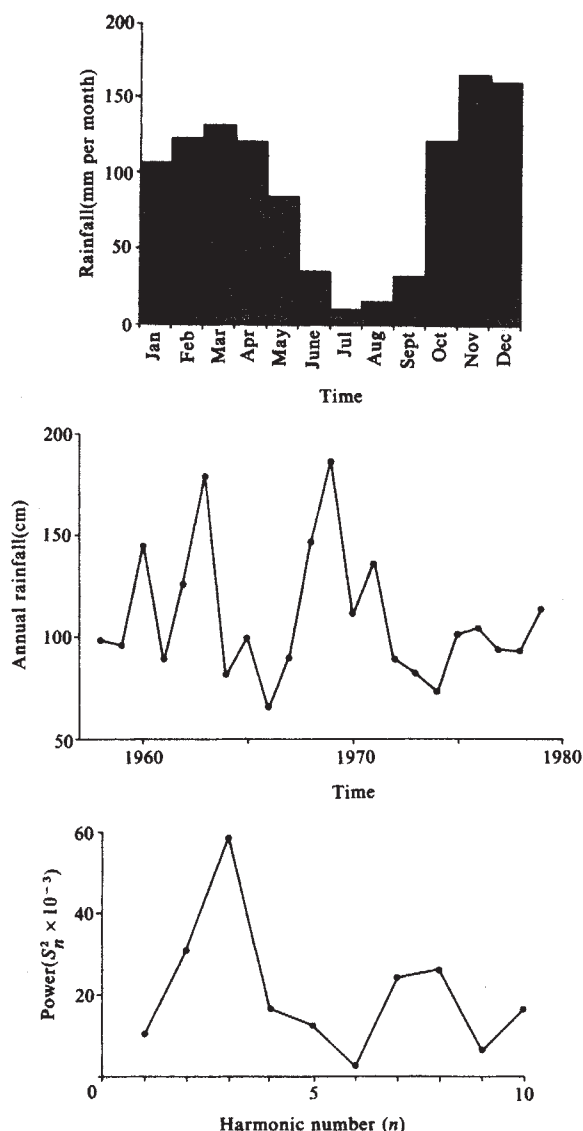


Fig. 4 a, The variation of monthly rainfall at Ifrane based on results from 1927 to 1949. b, The variation of annual rainfall at Ifrane from 1957 to 1979. c, The Fourier power spectrum of the annual rainfall at Ifrane from 1957 to 1979.

Modern archaeomonads indicate sea-ice environments

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Diatoms, radiolarians, silicoflagellates, ebridians and archaeomonads are the primary groups of pelagic organisms in siliceous marine deposits, with only the archaeomonads regarded as being absent from modern oceans and sediments. They remain a poorly known group of siliceous nanofossils in Cretaceous to Upper Tertiary marine sediments. We present here the first report, from any modern oceanic environment, of pelagic cysts that conform to the descriptions for Archaeomonadaceae. These cysts occurred in seawater, sea ice and marine-surface sediments, demonstrating the group is still extant. Furthermore, their distribution in modern sediment suggests they may be useful guide fossils for sea-ice environments as they occur abundantly only in polar or near-polar water where sea ice is present. Few other microorganisms seem to have such a close and exclusive association with sea ice, thus archaeomonads deserve increased attention as indicators of ice conditions for deep-water marine environments.

Small (7–20 µm) siliceous cysts found as minor constituents of many Upper Cretaceous to Pleistocene sediments, archaeomonads dominate few sedimentary assemblages^{1,2}, and thus their environmental affinities are poorly known. First described as a taxonomic group^{3,4}, archaeomonads are generally considered to represent resistant stages in the life histories of unknown or extinct marine chrysophytes, as they closely resemble the statocysts produced by freshwater chrysophytes^{5–9}. Both archaeomonads and chrysophyte cysts are siliceous spheres possessing a single pore that is usually surrounded by a flange. Surfaces vary from smooth to spiny and are important taxonomic features in both taxa. Pelagic chrysophyte cysts have been reported from oceanographic surveys in the Weddell Sea¹⁰ and other areas¹¹, but little or no taxonomic, descriptive or morphological information is given in these reports, making it impossible to determine whether the cysts belong to the Archaeomonadaceae or some other group such as those described by Silver *et al.*¹². Moreover, previous studies have relied on light microscopic examination of material, but careful descriptions of the diagnostic features of these nanoplankton-sized organisms require scanning electron microscopy (SEM).

Fifty-three water samples were obtained from a 10,000-km² area centred around 74°27'S, 37°53'W during the 1978 International Weddell Sea Oceanographic Expedition (IWSOE)¹³. The samples were taken at depths of 2–108 m; temperatures of –1.844 to –1.697 °C; oxygen concentrations of 6.85–8.79 ml per l, salinities of 34.01–34.94‰, and bottom depths of 377–1,982 m. All samples were taken at least 50 km from land or glacier in a water column averaging 500 m in depth. Water samples were filtered through Nuclepore filters (0.4 µm pores) and oven dried at 50 °C. This sample processing method destroys cellular components, making it impossible to study the vegetative cells associated with cyst hard parts, but leaves skeletal material clean and unharmed.

To prepare the filtered specimens for SEM, filters were coated with gold and examined using a JEOL JSM2 scanning electron microscope. To determine the wall composition, individual archaeomonads were analysed by X-ray fluorescence in a Cambridge S-180 SEM with an ETEC EDAX attachment. Counts were made across filters during routine SEM using transects of known area.

Twenty-three of the Weddell Sea water samples examined contained archaeomonads. Their average abundance was $1.4 \times 10^3 \text{ l}^{-1}$ in the seawater samples, (s.e. 2.8×10^2 , the maximum was $9.4 \times 10^3 \text{ l}^{-1}$). Archaeomonads were found in 20 out of 29 samples from 0 to 55 m and in 3 out of 24 samples from 55 to 110 m. Archaeomonads abundance decreased significantly with depth ($P < 0.01$). Four species were found in the Weddell Sea water column, *Archaeomonas areolata*, *A. formosa*, *A. reticulosa* and *Littheusphaerella spectabilis*. The X-ray analysis confirmed that specimens identified as archaeomonads possessed walls of silicon.

Additional records of archaeomonads in water samples were obtained from a search of our data files on epipelagic organisms found in the central California Current region and from a 1,650-m water sample (provided by A. Alldredge) from the same area. These records indicate that archaeomonads occurred in two locations from the central California Current at 35°70'N, 123°67'W, 50 m and 32°N, 118°W, 1,650 m (M.W.S. unpublished data). Although very rare in these samples, two different species were present, *A. mangini* and *A. striata*.

Four sea-ice samples were taken from the same area as the Weddell Sea water samples, one during the 1978 IWSOE and

Table 1 Coordinates for locations shown in Fig. 1

Deep Sea Drilling Project sediment samples

Site	Lat.	Long.	Series	Depth
Leg 12				
112	54°01' N	46°36' W	Upper Pleistocene	3,657
Leg 18				
178	56°57' N	147°08' W	Upper Pleistocene	4,218
182	57°53' N	148°43' W	Upper Pleistocene	1,411
Leg 19				
183	52°34' N	161°12' W	Upper Pleistocene	4,708
186	51°07' N	174°00' W	Upper Pleistocene	4,522
188	53°45' N	178°40' E	Upper Pleistocene	2,649
190	55°33' N	171°38' E	Upper Pleistocene	3,875*
191	56°56' N	168°10' E	Upper Pleistocene	3,854
193	45°48' N	155°52' E	Upper Pleistocene	4,811
Leg 28				
266	56°24' S	110°07' E	Upper Pleistocene	4,167
268	63°57' S	105°09' E	Upper Pleistocene	3,529
269	61°41' S	140°04' E	Quaternary	4,282
270	77°26' S	178°30' E	Pliocene–Recent	663*
271	76°43' S	175°03' W	Quaternary	562*
272	77°08' S	176°46' W	Lower Pliocene	619*
273	74°32' S	174°38' E	Quaternary–Pliocene	491*
Leg 35				
323	63°41' S	97°58' W	Upper Pliocene	5,004
325	65°03' S	73°40' W	Upper Pliocene	3,748
Leg 36				
326	56°35' S	65°18' W	Upper Pleistocene	3,812
327	50°52' S	46°47' W	Upper Pleistocene	2,400
328	49°49' S	36°40' W	Upper Pleistocene	5,095
Leg 38				
340	67°12' N	06°18' E	Upper Pleistocene	1,217
344	76°09' N	07°52' E	Pleistocene	2,156
348	68°30' N	12°28' W	Upper Pleistocene	1,763

Scripps Institution of Oceanography repository sediment samples

Lat.	Long.	Depth (m)
50°49' N	166°35' W	5,025
12°37' S	78°39' W	5,960
14°18' S	78°18' W	4,300
17°38' S	16°12' W	3,427
48°13' N	157°26' W	5,220
51°34' N	149°58' W	4,484
0°55' N	104°12' W	3,496
1°00' N	113°42' W	3,947
1°01' N	124°33' W	4,771
0°59' N	135°05' W	4,297
64°03' S	61°39' W	1,050*

* Archaeomonads present.

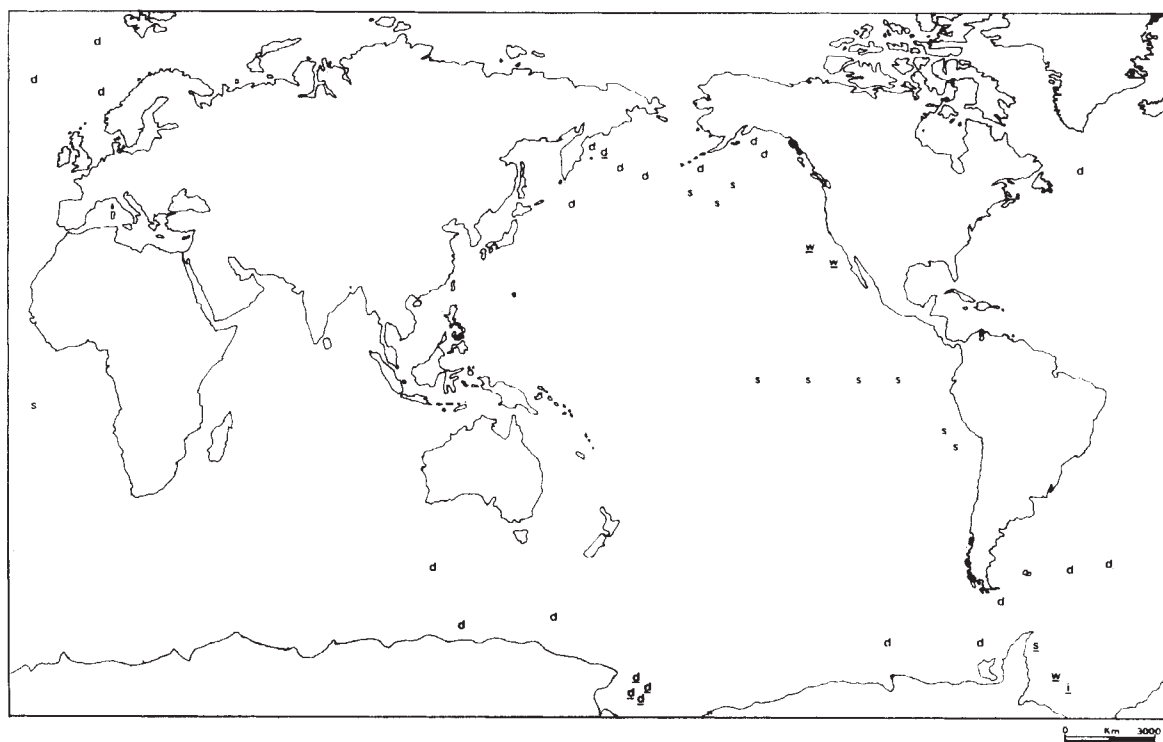


Fig. 1 The distribution of samples examined. d, Deep Sea Drilling Project samples; s, Scripps Institution of Oceanography samples; w, water column samples; i, ice samples. Underlined letters indicate the locations where archaeomonads were present.

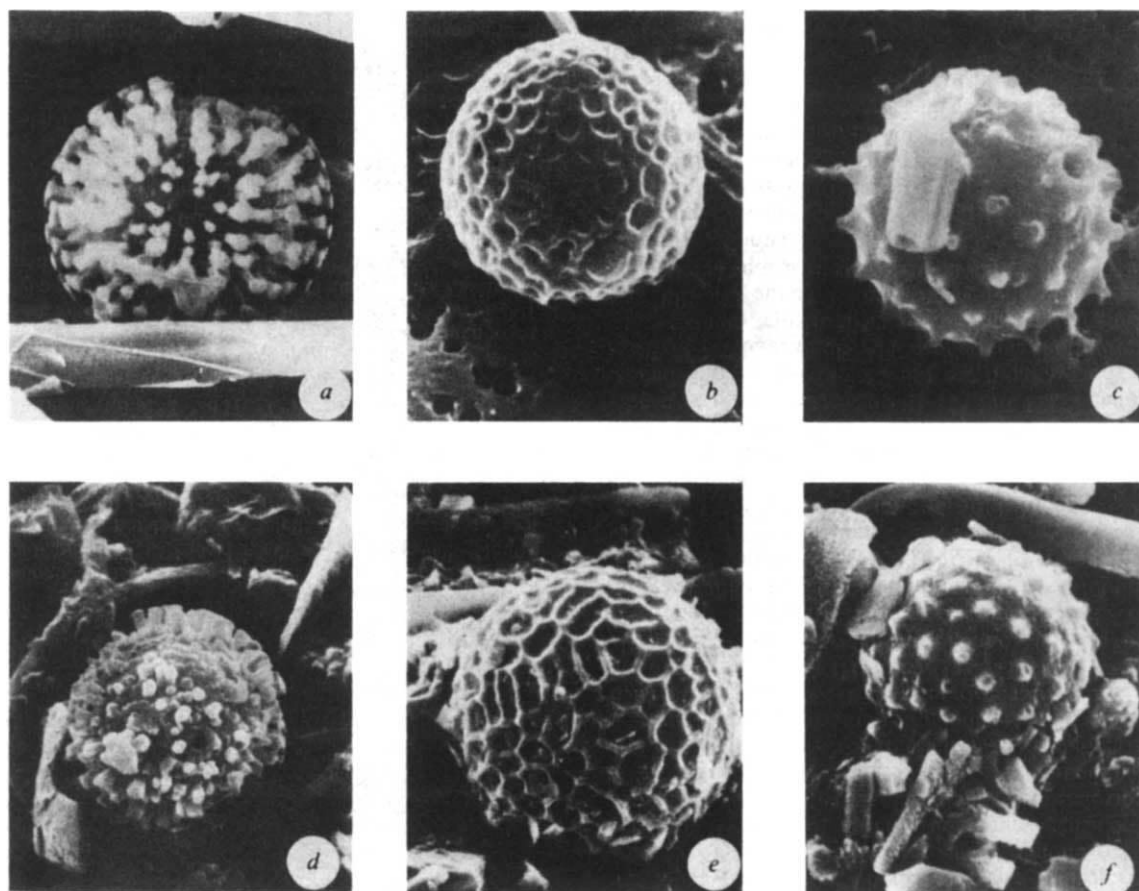


Fig. 2 *a-c*, Archaeomonads from sea-ice and water samples. *d-f*, Archaeomonads from sediment samples. *Litheusphaerella spectabilis* Deflandre (*a* and *d*). *Archaeomonas areolata* Deflandre (*b* and *e*). *A. mangini* Deflandre (*c* and *f*). Other archaeomonads tentatively identified to species were: *A. striata* Deflandre, *A. sphaerica* Deflandre, *A. reticulosa* Deflandre and *A. formosa* Deflandre. See the text for exact distributions. Perch-Nielsen^{16,17} and Deflandre⁶ provide species descriptions and scanning electron micrographs of the above species, and Stradner² the ultrastructural information on *A. areolata*. Magnifications $\times 6,500$ and background pores in *b* and *c* are $0.4\ \mu\text{m}$ in diameter.

three during the 1980 IWSOE. After the ice was washed and melted, the ice microorganisms were prepared in the same way as those from the water column. The 1978 sample was quantitatively prepared and counted, whereas those from the 1980 season were not; thus the 1980 material provides only qualitative data. Archaeomonads were present in all four samples of ice, and they occurred in densities of $13 \times 10^3 \text{ l}^{-1}$ in the 1978 material and also were abundant, relative to diatom frustules, in the 1980 samples. Four species were found in Weddell Sea ice from the two years, *A. areolata*, *A. formosa*, *A. mangini* and *L. spectabilis*.

Thirty-six samples of surface marine sediment were examined (Table 1, Fig. 1) from areas throughout the world ocean where siliceous sediments are presently being deposited¹⁴. Twenty-five sediment samples were obtained from the Deep Sea Drilling Project repository, and 10 from the core library at Scripps Institution of Oceanography. The remaining sample was from a gravity core taken in the Weddell Sea ($64^\circ 03' \text{ S } 61^\circ 39' \text{ W}$). Thirty-one sediment samples range from Upper Pleistocene to Recent; three sediment samples are Pliocene, and two samples were dated as no older than the Pliocene (Table 1).

Cysts were obtained by suspending sediment aliquots in vials of distilled water, dispersing the slurry using a vortex mixer, and allowing the coarser fraction to settle for 5–7 min after which the upper third of the water was decanted, filtered and the filter treated for SEM. To avoid bias in the results, the samples were processed so that during the examination we did not know the origin of the sediment.

Archaeomonads were found in 6 out of the 36 sediment samples examined (Table 1). Five of the six samples were from south of the Antarctic Circle. One came from the Weddell Sea, four from the Ross Sea and one sample from the Western Bering Sea ($55^\circ 33' \text{ N}$, $171^\circ 38' \text{ E}$). Six species of archaeomonad were found in the Ross Sea sediment samples, *A. areolata*, *A. formosa*, *A. mangini*, *A. reticulosa*, *A. sphaerica* and *L. spectabilis*. Two species were found in the Weddell Sea sediment sample *A. areolata* and *A. striata*. As the samples without archaeomonads were searched as carefully as those containing the cysts, we think the lack of these specimens in the other samples indicates a real decrease in their abundance outside the areas of sea ice.

The discovery of significant numbers of archaeomonads in sea ice, water underlying the ice, and surface sediments underlying areas often covered with ice (Fig. 2) represents the first finding of archaeomonad-like cysts in the modern ocean. Their appearance in these samples establishes continuity from early Pleistocene archaeomonads to Recent marine chrysophyte cysts, and the modern distribution implies a distinct association between modern archaeomonads and sea ice. Although these cysts occurred in low numbers elsewhere, as shown by their presence in the California Current water samples, they were insufficiently abundant to contribute to underlying sediments. Similar sea-ice associations have been reported by Sancetta¹⁵ who found particular abundance ratios of diatom species in sediments associated with overlying ice and hydrographic conditions. Although chrysophytes occur throughout the world's oceans, they may, like their freshwater relatives⁹, produce a significant number of cysts only in extreme environmental conditions.

The distribution and abundance data presented here suggest that, in response to sea-ice formation, significant numbers of chrysophytes form the resistant cysts described previously as archaeomonads. This response to sea-ice coverage, which is a limited stimulus on a global scale, may explain why archaeomonads are absent or rare in most siliceous sediments and suggests other locations where they might be found. The association of archaeomonad cysts with polar sea ice, indicates they may be useful palaeoclimatological indicators.

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Earliest floral evidence for the Ebenaceae in Australia

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Flowers are seldom preserved in an identifiable condition. The limited knowledge of floral structure of ancient flowering plants makes comparisons with living plants difficult, as floral structure is the basis for classification of the angiosperms. When found, fossil flowers contribute considerably to our understanding of the evolution of flowering plants^{1–6}. The flowers described here were recovered from Upper Eocene deposits near Anglesea, Australia. They are tetramerous, unisexual, male, with 16 stamens and tricolporate pollen. These flowers, and associated ebenaceous leaves, are the oldest known remains of the Ebenaceae from Australia.

The Ebenaceae, the persimmon and ebony family, represents one of the small group of families of angiosperms whose name is not based on a currently valid generic name. The familial name was derived from the pre-Linnaean *Ebenus* Rumphius. Generic limits within the family are the subject of some controversy. As many as nine genera, including the widely used *Maba* Forst., *Royena* L. and *Tetraclis* Hiern have been recognized, but a more recent consensus has included all but one of these, *Euclea* Murr., in *Diospyros* L., the currently valid type genus^{7–9}. Of the 400–500 species ascribed to the Ebenaceae, only 14 may be attributed to *Euclea*, with the remainder in *Diospyros* sensu lato. Generic segregation was rejected due to the great uniformity of floral structure throughout *Diospyros* s.l.

The Ebenaceae is pan-tropical, with a few temperate outliers such as the American persimmon, *Diospyros virginiana* L. By far the greatest diversity is found in the Indo-Malaysian region. No recent synopsis of Australian species is available, but published regional floras indicate that 15 species of *Diospyros* occur

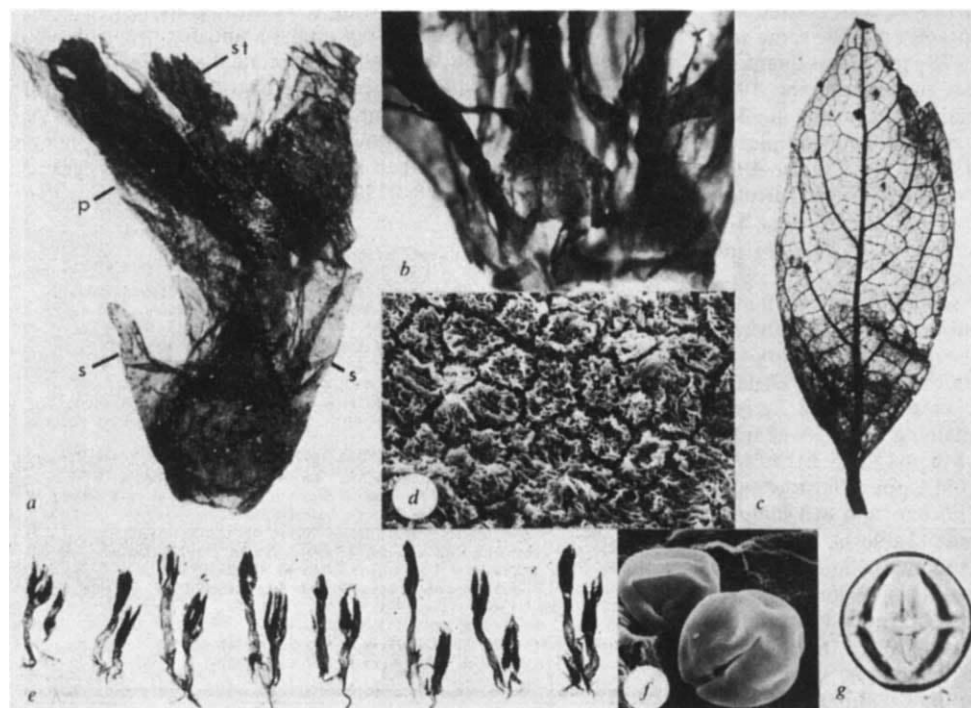


Fig. 1 Fossil flowers and leaves from the Eocene of Anglesea, Australia. *a*, Complete flower showing sepals (*s*), petals (*p*) and stamens (*st*), specimen number A-340 in the University of Adelaide Paleobotanical Collection ($\times 7$). *b*, Rudimentary gynoecium, surrounded by filaments of stamens, at centre of flower, A-342 ($\times 25$). *c*, Fossil leaf found in association with flowers, AM-243 ($\times 1.4$). *d*, Scanning electron micrograph of abaxial surface of leaf; note cuticular thickenings over epidermal cells and the overarching papillae that obscure the stomata, AM-260 ($\times 365$). *e*, Stamens removed from a complete flower, arranged in their relative position within the flower; note that stamens occur in pairs with one short and one long filament, A-341 ($\times 5$). *f*, Scanning electron micrograph of a fossil pollen grain, in polar view, preserved within an anther; note the smooth appearance of the exine ($\times 450$). *g*, Light micrograph of a fossil pollen grain from an anther; equatorial view showing tricolporate structure ($\times 450$).

in Australia^{10,11}. Although many stellate calyces and entire-margined leaves of late Cretaceous, and Tertiary age have been assigned to *Diospyros*, in few instances are features well enough preserved or of sufficient diagnostic value for positive identification. The fossil record for leaf and especially floral structure of the Ebenaceae is scant.

The fossils described here were found in clays overlying the Eastern View Coal Measures exposed at the Alcoa power station at Anglesea, Victoria. The sequence includes coarse to fine cross-bedded sands with lenses of carbonaceous clay, and has been dated as late Eocene^{12,13}. Most of the ebenaceous fossils occur in a single clay lens in association with a diverse leaf flora of over 40 taxa. Previous studies of clay lenses about 8 m higher in the sequence have revealed the presence of Casuarinaceae, *Bowenia*, and a zamoid cycad, *Pterostoma Hill*¹⁴⁻¹⁶.

Approximately 100 flowers have been recovered. All that remains of most of them are four-parted calyces whose sepals are fused in their basal third. Total sepal length is 3–5 mm and width is ~2 mm, and their shape is roughly triangular. Sepals possess numerous simple hairs on both inner and outer surfaces. The corolla and sexual organs seem to have been lost from the calyx as a unit. This is supported by the presence of such units in the macerates. Complete flowers and isolated corolla units contain only stamens and were strictly unisexual, male flowers. A small, bulbous structure at the centre of a few flowers represents an aborted gynoecium (Fig. 1*b*).

The actinomorphic corolla consists of four contorted petals that alternate with the sepals. The petals are fused to just above the tips of the sepals, a distance of about 1/3 of their total length of 7–9 mm. Petals, like sepals, have unicellular, simple hairs, although hairs tend to be most abundant on the margins and abaxial midline.

The androecium consists of 16 stamens which are paired, each pair comprising one long- and one short-filamented anther (Fig. 1*e*). Each anther has four locules and dehisces by longitudinal slits. Bleaching of the fossil anthers often causes separation of the tapetum from the anther wall, freeing four pollen-filled pouches from each. The pollen is tricolporate, subprolate, and ~30 μ m in equatorial diameter (Fig. 1*f, g*). The grains have a smooth to slightly scabrate exine.

No female flowers have yet been recovered. However, the observed rudimentary gynoecia are sufficient to illustrate that

the flower is hypogynous, with an ovary with more than one style. Unisexuality in flowering plants is commonly achieved by such incomplete development of one type of sex organ. The clustering of well-preserved fossil flowers may indicate that they are derived from one or a few individuals that grew very close to the site of deposition. Most living *Diospyros* are dioecious, and a similar condition in the fossil species may explain the abundance of male and apparent lack of female flowers.

Floral and pollen morphology of the fossil flowers form a unique suite of characters found only in one extant family, the Ebenaceae. Favourable comparisons may be made with many extant species of *Diospyros* s.l. *Diospyros* is a closely knit genus with remarkably uniform floral structure. Although flowers are tri-, tetra-, to pentamerous, the general appearance of the unisexual flowers, with their calyx cup, connate and contorted petals, radial pairs of stamens, tricolporate pollen, and superior ovary, varies little. The presence of eight radial pairs of stamens in the fossil and in many tetramerous extant *Diospyros* is very unusual among flowering plants and in itself could be considered diagnostic. Only minor differences in shape and size of floral organs, and no significant structural differences, distinguish the fossil flowers from such living tetramerous species of *Diospyros* as *D. australis* Hiern.

Associated with the fossil flowers are leaves of more than 40 species of angiosperms. The commonest of these, currently represented by 45 complete individuals in our collection, is a

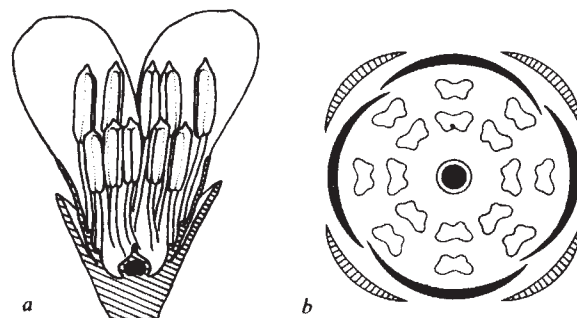


Fig. 2 *a*, Reconstruction of flower in median longitudinal section. *b*, Floral diagram.

highly variable taxon. It ranges in size from 4 to 110 mm in length and 2 to 60 mm in maximum width. Shape is equally variable, ranging from lanceolate to ovate to nearly orbicular. Venation is brochidodromous (Fig. 1c). These features are consistent with several extant Australasian species of *Diospyros*, but are by no means diagnostic of them. Of greater importance is the cuticular structure of these fossil leaves. The stomatal complexes are of the actinocytic type, with 4–6 irregular subsidiary cells. Each subsidiary cell produces a large papilla that overarches the guard cells. Cells of the lower epidermis possess extensive ridge-like thickenings of the cuticle (Fig. 1d).

Cuticular patterns on the abaxial epidermis of extant *Diospyros* are extremely diverse, ranging from nearly smooth (such as *D. calycantha* O. Schwarz), to striate or wrinkled, to ridged, to ornate, with large, frill-like accumulations of cutin (such as *D. australis* Hiern, *D. macrocarpa* Hiern). Due to intraspecific consistency of characteristic epidermal modifications, cuticular analysis is a valuable tool in identification and classification of both living and fossil plants. Cuticular thickenings, subsidiary cell arrangement, and over-arched stomata of the fossil leaves are similar to many extant *Diospyros* and fit easily within the range seen in the genus.

Of great significance is the occurrence of the same distinctive cuticular pattern on the fossil leaves and on the sepals of the fossil ebenaceous flowers. Flowers and leaves have not been found in organic connection. However, close association in the fossil flora, similarity of foliar and floral features to living Ebenaceae, and especially cuticular similarity of both organs indicate that the fossil leaves and flowers represent one taxon.

The evolutionary history of the Ebenaceae is unclear. There is a general consensus that the Ebenales was derived from the Theales, but the divergence has been significant^{17,18}. Many fossils (both leaves and fruits) have been ascribed to *Diospyros*. They are reported from North America, Europe, and Asia as well as Australia^{19–22}. Ettingshausen reported *Diospyros* leaf impressions and fruit casts from Australia²³. However, with no floral structure and no cuticles, his identifications are questionable. Similarly, serious doubt is cast on other reports of macrofossil evidence of the family. Ebenaceous pollen is not distinctive, and Palaeogene reports are unreliable. The flowers reported here are the earliest known identifiable floral remains of the Ebenaceae and the earliest well documented occurrence of the family in Australia.

As the Ebenaceae is pan-tropical, with the centre of diversity in Malaysia, it is tempting to believe that this might have been the region of origin. If this were true, the 15 extant Australian species would most probably have evolved from post-Miocene arrivals through New Guinea, since Palaeogene dispersal from South-east Asia to Australia would invoke excessive, if not incredible distances.

A Malaysian origin is opposed by the many species in Australia, South America, and Africa, and the Eocene fossils from Australia, which suggest that the Ebenaceae had a Gondwana origin with subsequent dispersal to the Malaysian region, possibly as late as the Miocene, through India and/or Australia. However, if some of the Laurasian fossils of early Tertiary age, in particular flowers assigned to *Diospyros* from the British late Eocene²², are correctly assigned to the Ebenaceae, the family may have already become very widespread by that time.

The Anglesea fossils also provide evidence that the floral features typical of many Ebenaceae—for example, tetramery, unisexuality, stamens in pairs and four times the number of corolla lobes, fused and contorted petals, and tricolporate pollen—were well established by Eocene times, and that selective pressures since that time have not been sufficient to alter them. Foliar features, too, were well advanced. Leaf architecture and the peculiar cuticular thickenings that characterize living Ebenaceae were present in the Eocene and were already associated with a typically ebenaceous floral plan.

Systems of angiosperm classification continue to be based, as they have since the time of Linnaeus, on floral morphology.

The validity of phylogenetic interpretations based on these systems is tested most critically by floral evidence from the fossil record. Within this framework, the importance of the discovery and analysis of structurally preserved flowers, such as those from Anglesea, is obvious.

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Self-excitation of olfactory bulb neurones

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The concept that neurotransmitter released from a neurone may feed back and influence the excitability of the same neurone has been suggested by a variety of evidence. Anatomical studies have shown that axon collaterals can arborize among the dendrites of the parent neurone^{1–7}, suggesting a direct feedback via axon collaterals. In addition, the dendritic release of dopamine from substantia nigra neurones has suggested that dopamine may exert a direct feedback inhibition of these neurones^{8,9}. Little electrophysiological evidence is available, however, to indicate that such a mechanism does exist. Based on intracellular recordings, Park et al.¹⁰ have proposed a direct inhibitory feedback in the neostriatum, but a presynaptic mechanism was not entirely excluded. We have now found that blockade of synaptic inhibition of relay neurones in the olfactory bulb unmasks long-lasting depolarizing potentials which can trigger repetitive discharges. These depolarizing potentials result from direct feedback of dendritically released excitatory transmitter onto the same and neighbouring relay neurones. Such a process might contribute to epileptogenic neuronal discharge.

We have used the *in vitro* turtle (*Pseudemys scripta elegans*) olfactory bulb preparation^{11–13}. The bulb was hemisected to facilitate the placement of stimulating and recording electrodes. Microelectrodes (140–180 M Ω) filled with 2 M potassium methylsulphate were used to record intracellularly from mitral cells (the principal relay neurones of the olfactory bulb). Mitral cells could be activated synaptically by stimulating the olfactory

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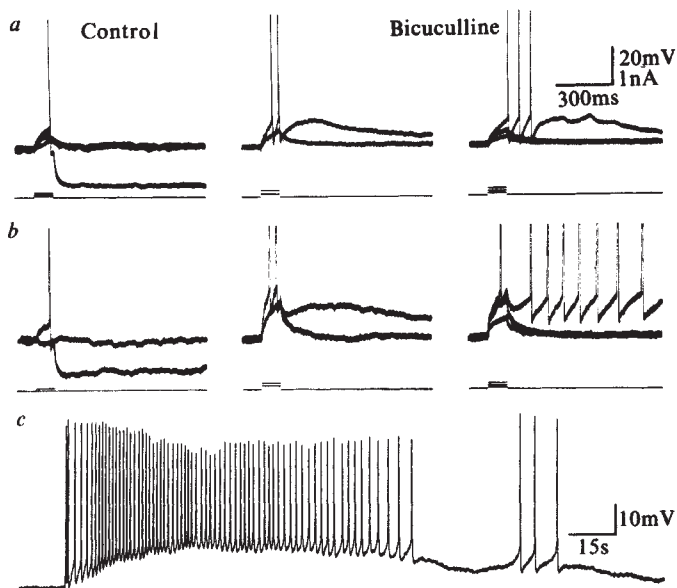


Fig. 1 Blockade of i.p.s.ps reveals depolarizing after-potentials. The superimposed oscilloscope records in this and the following figures show one or two traces with subthreshold depolarizing current pulses applied through the recording microelectrode and one trace with a suprathreshold pulse. The current trace is shown below the voltage trace. *a, b* Show two examples of the effect of bicuculline methiodide (BMI) (0.1 mM). The GABA antagonist had been present for 8 min during the second and third responses in *a*, and for 10 min in *b*. The cells often fired repetitively during the depolarizing after-potential. Resting membrane potential for cell in *a* is -57 mV and in *b*, -68 mV. Spikes in *b* are truncated. *c* Shows a pen record of a prolonged burst of action potentials elicited by a single 100-ms depolarizing current pulse. This cell had been exposed to BMI (0.2 mM) for 17 min. Resting membrane potential -52 mV. The calibration in *a* also applies to *b*.

nerves, antidromically by stimulating their axons or directly by passing depolarizing current pulses through the recording electrode. A bridge circuit was used so that the membrane potential could be monitored during the current pulses.

In the absence of an antagonist of γ -aminobutyric acid (GABA), action potentials evoked in mitral cells by the injection of depolarizing current were followed by inhibitory postsynaptic potentials (i.p.s.ps) (Fig. 1*a, b*) which result from the direct activation of the reciprocal dendrodendritic synapses between mitral and granule cell. Similar to the i.p.s.ps elicited by stimulating mitral cell axons¹²⁻¹⁴, these directly activated i.p.s.ps are thought to be mediated by GABA^{12,13}. Addition of the GABA antagonists, bicuculline methiodide or picrotoxin, to the superfusate blocked the hyperpolarizing i.p.s.p. and, in 40 of 46 cells examined, uncovered a prolonged depolarizing after-potential (DAP) (Fig. 1*a, b*) which was at times intense enough to trigger repetitive firing of the mitral cell (Fig. 1). In Fig. 1*c*, a single 100-ms depolarizing current pulse evoked a discharge lasting for ~ 3 min. We considered three possible mechanisms for this DAP. It could result from an increase in a sodium conductance which inactivates slowly, such as has been reported in cerebellar Purkinje cells¹⁵. However, unlike the Purkinje cell response, the mitral cell DAP was not blocked by the addition of a concentration of tetrodotoxin (TTX) ($1 \mu\text{M}$) sufficient to block the fast directly evoked action potential ($n = 11$) (Fig. 2).

Another process which could produce the DAP is a slow, voltage-dependent calcium conductance. The DAP is calcium dependent because in the seven cells examined it was blocked by the calcium antagonists cobalt (2 mM) or cadmium (0.5 mM), which also blocked the TTX-insensitive action potential (Fig. 2; see also refs 11-13). The calcium antagonists would also be expected to block the release of transmitter. If the DAP were a result of a voltage-dependent mechanism (whether calcium or sodium mediated) it should be aborted by hyperpolarizing

the membrane, as has been seen in other systems¹⁶⁻¹⁸. However, injection of a hyperpolarizing current pulse through the recording microelectrode did not alter the duration of the DAP ($n = 6$) (Fig. 3*a*) even when the hyperpolarizing pulse outlasted the DAP. This finding suggests that the DAP may result from feedback of excitatory transmitter released from the same mitral cell.

Another manipulation that might distinguish between a calcium conductance increase and a feedback of excitatory transmitter is the substitution of barium for calcium. Barium can readily pass through calcium channels (in some cases better than calcium¹⁹), but it substitutes poorly, if at all, for phasic evoked transmitter release²⁰⁻²³. We have tested the ability of barium to substitute for calcium in synaptic transmission in the olfactory bulb by recording the field potential evoked by the excitatory synaptic action of mitral cell dendrites on granule cell dendrites. We found that when synaptic transmission was blocked in a zero calcium Ringer, 3 mM barium was unable to restore transmission. However, switching back to normal Ringer containing 3 mM calcium quickly restored the synaptic potential. The effect of barium substitution on the DAP was examined in five cells. A typical result is shown in Fig. 3*b* in which the DAP was completely blocked while the amplitude of the TTX-insensitive spike was actually increased. The abolition of the DAP by barium substitution suggests that the DAP is mediated by the release of synaptic transmitter. Note that addition of barium to normal Ringer did not block the DAP¹³, indicating that barium is not a calcium antagonist for the release process, but rather fails to mimic the action of calcium. In light of the evidence presented above and because the DAP was not blocked by deleting axonal pathways with TTX, we suggest that mitral cell activation causes dendritic release of an excitatory transmitter which feeds back directly onto the same mitral cell.

Recent biochemical evidence strongly suggests that aspartate is the transmitter released from mitral cell axon terminals in the olfactory cortex²⁴. It is, therefore, reasonable to expect that aspartate is the transmitter released from mitral cell dendrites. We have found that iontophoresis of either glutamate or aspartate in the presence of GABA antagonists depolarizes mitral cells²⁵. α -Amino adipate (α AA), which antagonizes aspartate responses in other systems^{26,27}, decreased the depolarizing

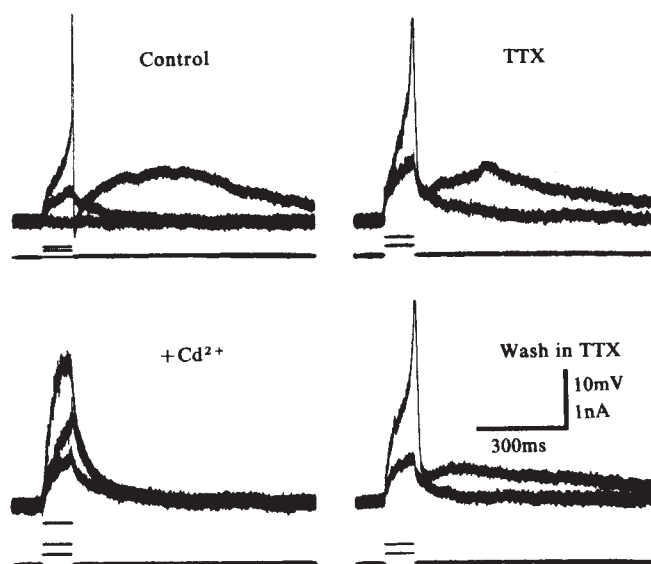


Fig. 2 The depolarizing after-potential is resistant to tetrodotoxin (TTX) but blocked by the calcium antagonist cadmium. The control record was obtained 10 min after switching to bicuculline (0.1 mM) (spike truncated). TTX ($1 \mu\text{M}$) was then superfused for 22 min and only slightly reduced the DAP. After exposure to cadmium (Cd^{2+}) (0.5 mM) for 10 min, the depolarizing after-potential and the calcium spike were entirely blocked. Washing for 90 min (in TTX) restored the calcium spike and DAP. Resting membrane potential, -56 mV.

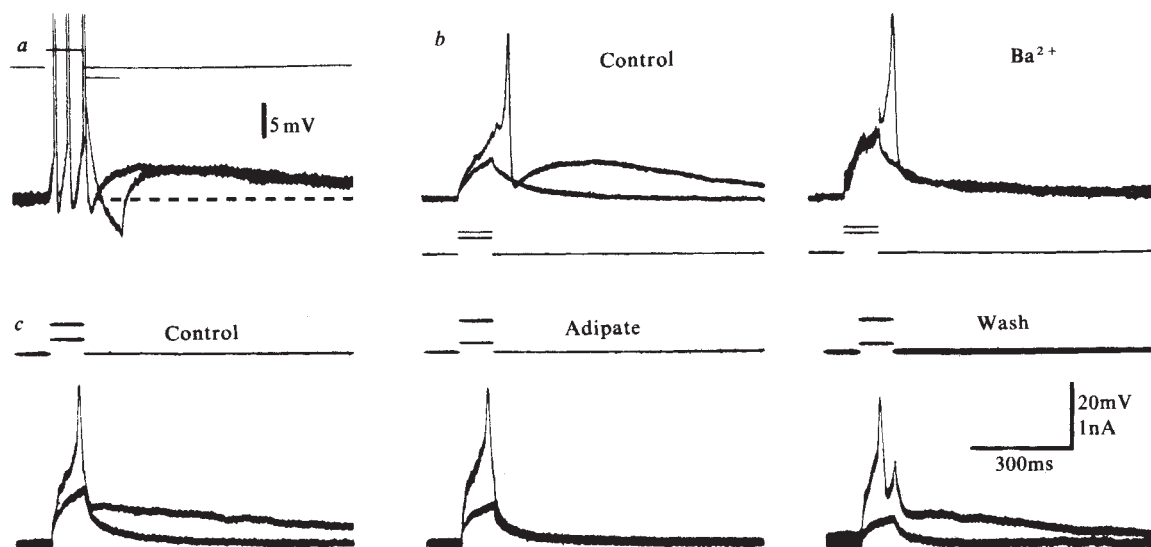


Fig. 3 Properties of depolarizing after-potential. In *a*, a hyperpolarizing current pulse applied immediately after a suprathreshold depolarizing current pulse did not diminish the DAP. The preparation had been bathed in BMI (0.1 mM) for 23 min. Resting membrane potential, -53 mV. The control record in *b* was obtained 30 min after switching to a solution containing TTX ($1 \mu\text{M}$) and BMI (0.1 mM). The record to the right was obtained 12 min after substituting 3 mM barium for calcium. Resting membrane potential, -57 mV. The control record in *c* was obtained 50 min after switching to a solution containing TTX ($1 \mu\text{M}$) and picrotoxin (0.2 mM). Adipate (1 mM) was then added to the superfusion medium for 15 min. The record on the right was obtained 24 min after returning to the control solution. Resting membrane potential, -62 mV. The calibration in *c* also applies to *b*.

effect of iontophoretic aspartate on mitral cells (not shown). We examined the sensitivity of the DAP to α AA in the presence of TTX so that we could use the calcium spike as a monitor of the specificity of α AA. The DAP was reversibly abolished by α AA ($n = 6$), which had little or no effect on the calcium spike (Fig. 3c). The blocking effect of α AA, therefore, occurred after calcium entry. The i.p.s.p. in normal Ringer was also reversibly abolished by α AA.

After inhibition was blocked, antidromic action potentials were also followed by DAPs (Fig. 4b). The antidromic spike can be fractionated by passing a hyperpolarizing current pulse through the recording microelectrode, timed to occur when the antidromic spike arrives at the soma^{12,13}. Large current pulses blocked invasion of the soma-dendritic membrane, revealing a small action potential that probably resides in the initial segment^{28,29}. When only the initial segment was activated, the resulting DAP was small (Fig. 4b). However, when invasion of the soma-dendritic membrane occurred, a larger DAP resulted. This incremental increase in the amplitude and duration of the DAP is, therefore, due to activation of the soma-dendritic membrane. A depolarization could also be evoked by antidromic stimulation which was subthreshold for the axon of the impaled cell (Fig. 4a). These findings suggest that transmitter released from antidromically invaded mitral cells can diffuse to neighbouring mitral cells. Indeed, in the presence of GABA antagonists, mitral cells frequently fire spontaneous bursts of action potentials (Fig. 4c), suggesting the presence of synchronized activity.

Thus, in normal conditions an excitatory transmitter, probably aspartate, is released from mitral cell dendrites and activates receptors on the processes of granule cells. The ensuing depolarization results in the release of GABA from the granule cells back onto the same mitral cell. When the inhibitory half of the reciprocal synapse is blocked with GABA antagonists, the activation of olfactory bulb mitral cells is followed by an excitatory depolarizing potential instead of an i.p.s.p. The present findings indicate that this depolarizing potential results from the direct feedback of dendritically released excitatory transmitter onto the same mitral cells. The depolarization elicited by both subthreshold antidromic stimulation and in the presence of only an initial segment spike indicates that this phenomenon is not limited to single cells, but can involve an interaction of

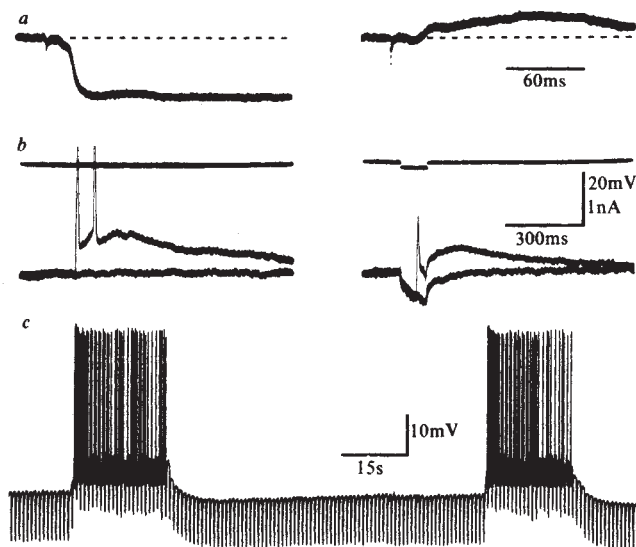


Fig. 4 Antidromic stimulation evokes depolarizing responses in the presence of GABA antagonists. The response on the left in *a* shows an i.p.s.p. to a stimulus below threshold for the axon of the impaled cell. The right-hand record was obtained 32 min after switching to a solution containing 0.2 mM picrotoxin. Resting membrane potential, -64 mV. The responses in *b* were obtained 12 min after switching to a solution containing 0.1 mM bicuculline methiodide. The record on the left in *b* shows the response to antidromic stimulation suprathreshold for the impaled cell (spikes truncated). The record on the right shows that when a hyperpolarizing current pulse blocked firing of the soma-dendritic membrane, the DAP was reduced in size but was still present. Resting membrane potential, -58 mV. *c* Shows spontaneous bursts of action potentials from the same cell illustrated in *a*, 47 min after switching to the picrotoxin-containing solution. The voltage gain in *a* is twice that in *b*.

a population of neurones, each contributing to the depolarization of its neighbours. In addition, excitatory recurrent collaterals could also contribute to this lateral spread of excitation³⁰. The prolonged depolarization which follows olfactory nerve stimulation in the presence of GABA antagonists^{13,31}

may also be due in part to similar excitatory feedback mechanisms. While the physiological significance of self-excitation, either via a dendritic or axon collateral pathway, is unclear, such a process might well contribute to epileptogenic burst discharge in other brain regions.

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Unique response to antipsychotic drugs is due to absence of terminal autoreceptors in mesocortical dopamine neurones

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A projection of dopamine (DA) neurones has been identified which runs from the rat midbrain ventral tegmental area to the cerebral cortex¹. Relative to other cortical areas, the prefrontal cortex is enriched in DA^{2,3}, tyrosine hydroxylase³, DA uptake^{2,3}, DA-sensitive adenylate cyclase² and DA-responsive neurones⁴. Lesions of the prefrontal cortex or selective lesions of the prefrontal cortical DA innervation induce functional deficits, including detrimental effects on delayed response tasks, a difficulty in suppressing attention to irrelevant stimuli and a diminution in affective and social behaviour (for a review, see ref. 5). Recent studies have shown that this mesocortical DA system differs from other DA systems (such as the nigrostriatal and mesolimbic DA systems) in terms of drug responsiveness. For example, the acute administration of antipsychotic drugs (for example, haloperidol) greatly accelerates DA turnover in the nigrostriatal and mesolimbic DA systems through multiple sites of action, but only modestly accelerates mesocortical DA turnover. Following the chronic administration of moderate doses of antipsychotic drugs, tolerance to the effects of a haloperidol challenge dose is generally seen in the striatum and olfactory tubercle but not in the frontal cortex^{6–8}. Similar results obtained in experiments involving primates have led to the suggestion that the frontal cortex may be the site of therapeutic

action of the antipsychotic agents^{9,10}. In light of the recent finding that rat mesocortical DA neurones lack terminal autoreceptors¹¹, we have investigated the possible role of nerve terminal DA autoreceptors in mediating the varied responses seen in different brain regions following acute and chronic antipsychotic drug treatment. The results, reported here, suggest that both the relatively small activation of the mesocortical DA system in response to a haloperidol challenge, and the lack of tolerance to haloperidol following chronic treatment, may be related to the absence of nerve terminal autoreceptors.

Male Sprague-Dawley rats (Charles River; 200–250 g) were injected intraperitoneally (i.p.) with either haloperidol (0.5 mg per kg) or vehicle for 28 days, as previously described¹². Some animals received a haloperidol challenge (1 mg per kg) or vehicle 60 min before decapitation. For experiments directly assessing DA autoreceptor function, other rats were withdrawn from haloperidol for 7 days before use. In other experiments, unilateral knife cuts of the medial forebrain bundle were performed under halothane anaesthesia 35 min before decapitation. The coordinates used were: 3,180 μ m anterior to lambda; 500 μ m lateral to the midline; 6,000 μ m ventral from the dura¹³. From this position the knife was extended ~4 cm and lowered to the base of the brain. The striatum, olfactory tubercle and prefrontal cortex were dissected from the brain¹¹, and DA and dihydroxyphenylacetic acid (DOPAC) levels determined using liquid chromatography with electrochemical detection^{11,14}.

Acute haloperidol challenge dramatically increased both striatal and olfactory tubercle DOPAC levels (Fig. 1). In the prefrontal cortex, a much smaller but significant increase in DOPAC was seen after haloperidol challenge (Fig. 1). In parallel experiments, haloperidol challenge greatly accelerated the α -methyltyrosine-induced decline of DA in the striatum and olfactory tubercle, but not that in the prefrontal cortex (M.J.B. and R.H.R., unpublished). It has been previously demonstrated that the small haloperidol-induced increase in prefrontal cortical DA synthesis is totally prevented following the blockade of dopaminergic impulse flow¹¹. As mesocortical DA neurones lack nerve terminal autoreceptors, it has been

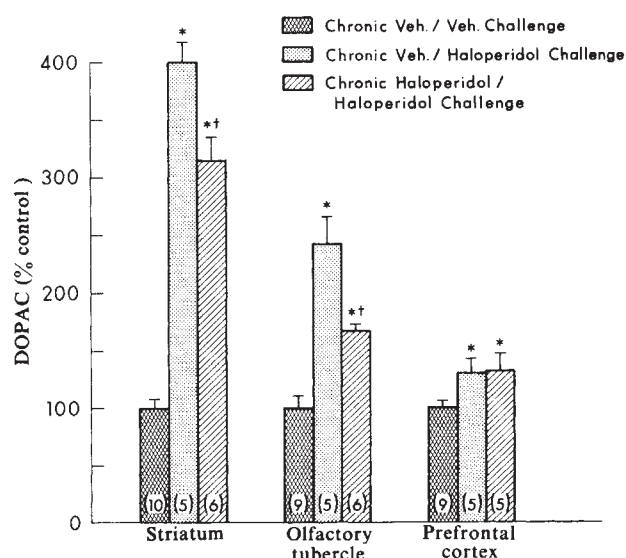


Fig. 1 Effects of acute and chronic haloperidol treatment on DOPAC levels in various brain regions. Animals were chronically treated with haloperidol or vehicle as described in the text. Haloperidol (1 mg per kg, i.p.) or vehicle challenge was administered 60 min before decapitation. The bars on the columns represent the s.e.m. and the numbers in the columns indicate *n*. Control striatal, olfactory tubercle and prefrontal cortical DOPAC levels were $1,092 \pm 86$, $1,004 \pm 96$ and 34 ± 2 ng per g respectively. * $P < 0.05$ when compared with vehicle-treated animals; ** $P < 0.01$ when compared with acute haloperidol challenge.

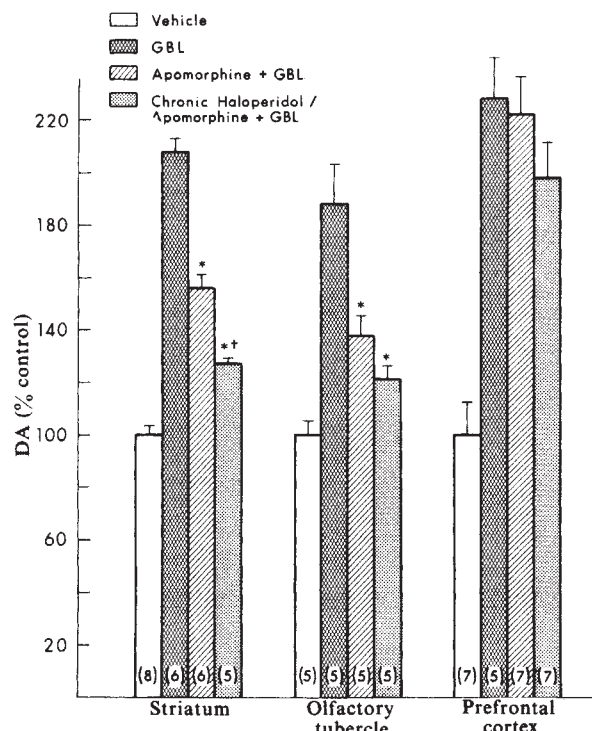


Fig. 2 Effects of chronic haloperidol treatment on DA autoreceptor sensitivity in various brain regions. Animals were chronically treated with haloperidol and withdrawn as described in the text. GBL (750 mg per kg, i.p.) was administered 35 min before decapitation. Some rats received apomorphine (0.25 mg per kg, i.p.) 5 min before GBL. The bars on the columns represent the s.e.m. and the numbers in the columns indicate *n*. Control DA levels in the striatum, olfactory tubercle and prefrontal cortex were $9,019 \pm 312$, $6,355 \pm 345$ and 86 ± 10 ng per g respectively. Chronic haloperidol treatment did not significantly alter DA levels (unpublished). * $P < 0.01$ when compared with animals treated with GBL alone; ** $P < 0.05$ when compared with the apomorphine-induced reversal of GBL in vehicle-treated rats.

suggested that the effects of haloperidol on this system are due solely to alterations in neuronal impulse flow¹¹.

Following chronic haloperidol treatment, significant tolerance to the DOPAC-elevating effect of a haloperidol challenge developed in both the striatum and the olfactory tubercle (Fig. 1). No change in the response to haloperidol was observed in the prefrontal cortex (Fig. 1). These results are in agreement with previous studies (in both rodents and primates) which have demonstrated the development of tolerance in the striatum, and in some cases limbic brain areas, with a lack of tolerance or even reverse tolerance in the frontal cortex⁶⁻¹⁰.

To examine the relationship between tolerance to haloperidol and changes in autoreceptor sensitivity, a pharmacological method for eliminating dopaminergic impulse flow was used. Administration of γ -butyrolactone (GBL) reversibly blocks DA impulse flow, resulting in an increase in tyrosine hydroxylation and DA levels in nigrostriatal and mesolimbic DA systems similar to that seen after mechanical lesioning of these systems (for a review, see ref. 15). These changes have been proposed to result from a decreased DA autoreceptor activation secondary to the decreased amount of DA released into the synaptic cleft¹⁶. Direct-acting DA agonists prevent this increase in DA synthesis solely through the stimulation of DA nerve terminal autoreceptors^{11,12}. As previously reported, the GBL-induced increase in striatal and olfactory tubercle DA was partially reversed by an intermediate dose of apomorphine (Fig. 2). The increase in prefrontal cortical DA was unaffected by apomorphine pretreatment (Fig. 2), indicating the lack of mesocortical nerve terminal autoreceptors, as previously described¹¹. Following chronic haloperidol treatment, an

enhanced response to apomorphine, that is, DA autoreceptor supersensitivity, was directly demonstrated in the striatum (Fig. 2), confirming previous results^{12,17,18}. The DA autoreceptors in the olfactory tubercle became slightly (but nonsignificantly) more sensitive, but no autoreceptor activity was revealed in the prefrontal cortex (Fig. 2).

To establish that the apparent lack of DA autoreceptors in the prefrontal cortex following GBL administration was not due to some unexpected effect of this drug on mesocortical neurones, autoreceptor function was assessed following the acute axotomy of the medial forebrain bundle (Fig. 3). Axotomy, like GBL, induced apomorphine-reversible increases in striatal and olfactory tubercle DA (Fig. 3), as previously reported^{16,19,20}. As expected, the axotomy-induced increase in prefrontal cortical DA was unaffected by apomorphine, providing additional evidence that mesocortical DA neurones lack terminal autoreceptors. The axotomy- and GBL-induced accumulation of prefrontal cortical DA (Figs 2, 3) are not a result of an acceleration of DA synthesis, but are presumably due to continued, normally rapid mesocortical DA synthesis for a period of time after the sudden blockade of impulse flow-coupled transmitter release^{11,21}.

DA neurones which lack nerve terminal autoreceptors show little (for example, the mesocortical system¹¹) or no (the tuberoinfundibular system²²) response to acute haloperidol challenge, while DA systems with terminal autoreceptors (for example, nigrostriatal, mesolimbic and tuberohypophyseal systems^{11,15,22}) respond dramatically. In the mesocortical system no tolerance to the effect of haloperidol challenge develops following chronic haloperidol administration, while tolerance does develop in the DA systems possessing nerve terminal autoreceptors⁶⁻¹⁰. Further, the time course for the development

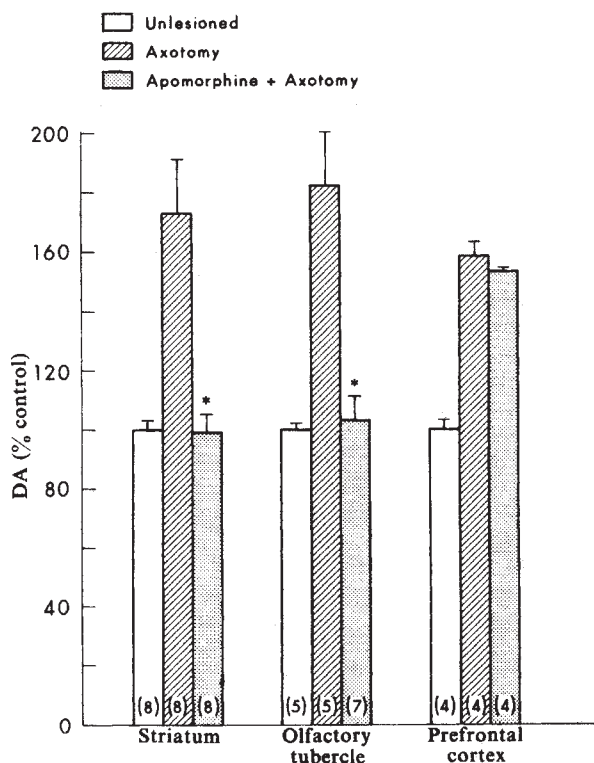


Fig. 3 Effects of apomorphine on nerve terminal DA autoreceptors after acute medial forebrain bundle axotomy. Lesions were made 35 min before decapitation using a retractable wire knife as described in the text. Some rats were pretreated with apomorphine (2 mg per kg, i.p.) 5 min before axotomy. The bars on the columns represent the s.e.m. and the numbers in the columns indicate *n*. DA levels in the unlesioned striata, olfactory tubercles and prefrontal cortices were $10,046 \pm 282$, $4,948 \pm 115$ and 87 ± 3 ng per g respectively. * $P < 0.001$ when compared with animals receiving axotomies alone.

of tolerance in these systems may parallel the development of autoreceptor supersensitivity. These results suggest that the acute and chronic effects of haloperidol and other antipsychotic drugs on the various DA systems may be related to the presence or absence of nerve terminal autoreceptors. In addition, it has recently been determined that, in contrast to nigrostriatal and mesolimbic DA cells, mesocortical DA neurones innervating the prefrontal cortex also lack cell body DA autoreceptors (B. S. Bunney and L. A. Chiodo, personal communication).

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The realization that mesocortical DA neurones lack both nerve terminal and cell body autoreceptors may aid in the development of potential therapeutic agents designed to interact selectively with discrete brain DA systems.

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Opioid peptides induce reduction of enkephalin receptors in cultured neuroblastoma cells

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Cultured mouse neuroblastoma cells (N4TG1)^{1,2} and neuroblastoma-glioma hybrid cells (NG108-15)³⁻⁵ contain enkephalin (δ) receptors². Studies on hybrid cells have indicated that occupation of enkephalin receptors by opiates and opioid peptides leads to a time-dependent inhibition of adenylate cyclase activity³⁻⁵. If exposure of these cells to agonists is continued, the activity of adenylate cyclase gradually returns to normal and increases above normal on removal of opioids, a phenomenon thought to resemble tolerance and dependence in animals. Direct receptor binding studies on neuroblastoma cells have shown no internalization of receptors¹. Using rhodamine-conjugated [D-Ala²,Lys⁶]Leu-enkephalin and image-intensified fluorescence microscopy, enkephalin (δ) receptors in neuroblastoma cells were found slowly to form clusters which do not appear to be internalized⁶. This is in contrast to many polypeptide receptors⁷⁻¹² which are internalized after the initial receptor-ligand interactions. This internalization is believed to be related to the so-called down-regulation or desensitization. We now report that after one to several hours exposure of monolayer cell cultures to enkephalin, the number of enkephalin receptors measured subsequently in the extensively washed membrane preparations is greatly reduced. This reduction in receptor number seems to be selective for opioid peptides and is antagonized by a variety of opiate agonists and antagonists. These observations may be important in elucidating the phenomenon of opioid-induced desensitization.

Confluent monolayers of N4TG1 cells were pretreated with ligand, the cells were then washed extensively with Dulbecco's phosphate-buffered saline (PBS) to remove any remaining bound ligand and membrane particulate fractions were prepared. Enkephalin receptor binding of these fractions was then determined by measuring binding of ¹²⁵I-labelled [D-Ala²,D-Leu⁵]enkephalin (DADLE). Pretreatment of the cell monolayer for 4 h with 0.1 μ M DADLE led to a large reduction in binding

of ¹²⁵I-DADLE to the subsequently prepared and washed membrane particulates. A similar loss of binding was observed for washed cells after pretreatment with DADLE; this reduction was time, temperature and dose dependent. At 37°C, a significant reduction was observed after 30 min pretreatment, and had almost reached a maximum after 1 h. Further incubation only slightly increased the reduction (Fig. 1). Routinely we preincubated for 4 h to ensure the maximum effect. At 24°C, the reduction in binding was greatly decreased with respect to both the rate and extent of reduction (Fig. 1). The DADLE-induced reduction of binding is dose dependent (Fig.

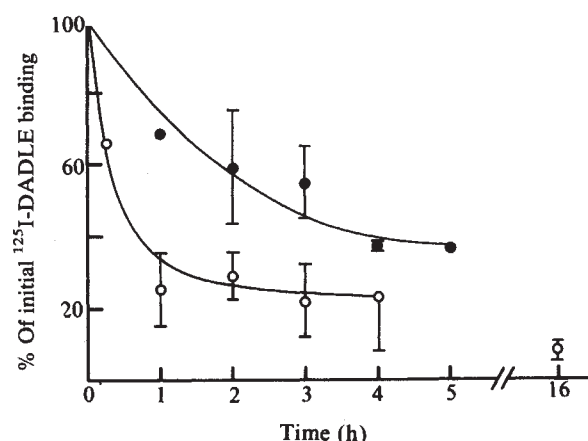


Fig. 1 Time course of the DADLE-induced decrease in ¹²⁵I-DADLE binding. N4TG1 cells were grown to confluence in Dulbecco's minimal essential medium supplemented with 75 U ml⁻¹ penicillin, 75 μ g ml⁻¹ streptomycin sulphate and either 7% or 5% newborn calf serum (Gibco) in a 95% air/5% CO₂ humidified atmosphere at 37°C. DADLE was added to a final concentration of 10⁻⁷ M and the monolayers were incubated at 37°C (○) or 24°C (●) for the indicated times. After completion of the incubation, the medium was withdrawn, the monolayers were rinsed with PBS and the cells detached using 0.05% EDTA in PBS. The cells were pelleted by centrifugation, resuspended in PBS and incubated at 37°C for 30 min to remove bound ligand. This incubation was repeated, then the cell pellet was resuspended in ice-cold 50 mM Tris pH 7.7 and the cells were homogenized using a Model PT-20 Polytron (setting 3.5, 20 s). The homogenates were centrifuged at 40,000g for 30 min and the resulting pellets were resuspended in 50 mM Tris pH 7.7 containing 100 mM NaCl; the suspensions were incubated at room temperature for 1 h. The membranes were again pelleted, resuspended and pelleted once in Tris buffer lacking sodium, and assayed for ¹²⁵I-DADLE binding as previously described^{1,2}.

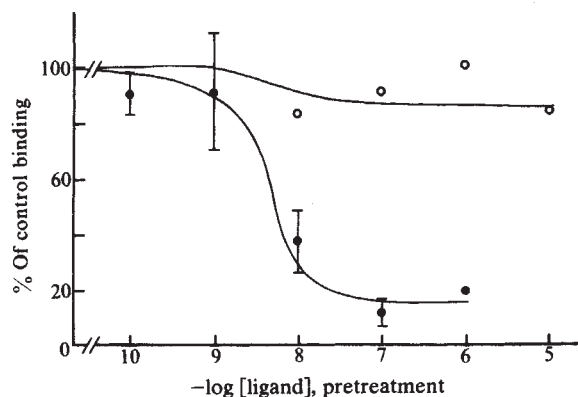


Fig. 2 Concentration dependence of the DADLE-induced decrease of enkephalin binding activity. Confluent monolayer cultures of N4TG1 cells were incubated with the indicated concentrations of DADLE (●) or morphine sulphate (○) for 4 h at 37 °C. Membrane fractions were prepared and binding activity was assayed as described in Fig. 1 legend. The data shown are the average values $\pm$ s.e.m. for two experiments.

2), having an EC_{50} (concentration which causes 50% of the maximum reduction) of ~ 2 nM. This value is in reasonable agreement with the previously determined dissociation constant (K_d) of DADLE for the enkephalin receptor of this cell line^{1,2}.

The reduction in 125 I-DADLE binding activity induced by DADLE is specific for opiate receptors as this effect can be antagonized by the presence of an excess of opiate antagonists such as naloxone, nalorphine and diprenorphine (Table 1). These antagonists do not induce by themselves a reduction in 125 I-DADLE binding activity (Table 1). The natural endogenous peptides, Met- and Leu-enkephalin, as well as β -endorphin, [D-Ala²,NMePhe⁴,Met(O)⁵]enkephalin and [D-Ala²,Met⁵]enkephalinamide, also induce a reduction in 125 I-DADLE binding. Morphine and benzomorphan drugs such as ketocyclazocine and *n*-allylnormetazocine at high concentrations not only induce just a minimal reduction (usually $\sim 20\%$) in the 125 I-DADLE binding activity (Table 1 and Fig. 2) but also antagonize the effect of opioid peptides (Table 1). This antagonistic effect of opiates against peptides is also dose dependent. Opiates are more effective against 0.01 μ M DADLE than against a dose of 0.1 μ M (Table 1), as would be predicted for competitive inhibition.

Incubation of cell monolayers with 10^{-8} M 3 H-DADLE at 37 °C for 4 h, followed by washing and preparation of membrane particulates in the same way as that used for the non-radioactive ligand, showed that 10% of the ligand initially associated with the cells remained bound. This corresponds to a concentration of DADLE $< 10^{-10}$ M in the final assay. Because incubation of cells with 10^{-8} M DADLE caused a reduction of $\sim 70\%$ in the 125 I-DADLE binding (Fig. 2), the loss of binding activity could not be attributed simply to the competition of 125 I-DADLE with ligand that remained after preparation of the membrane fragments. Furthermore, diprenorphine, a ligand known to have an affinity greater than that of DADLE¹³, did not cause receptor reduction after similar pretreatment, suggesting that the peptide-induced receptor loss could not be explained simply by the high affinity of any ligand not washed away.

The enkephalin-induced reduction in binding is manifested by a decrease in the number of high-affinity binding sites, presumably enkephalin (δ) receptors. The saturation binding curves of 125 I-DADLE for control and 0.1 μ M DADLE-pretreated cell membranes showed a loss of total high-affinity binding sites (Fig. 3). The binding sites remaining after DADLE pretreatment had affinity similar to that of the control membranes. This was further confirmed by 125 I-DADLE competition binding studies using DADLE and morphine, in which we observed no significant difference in IC_{50} values (concentrations

causing 50% inhibition of labelled binding) between control and pretreated membranes. As estimated from plots of log per cent 125 I-DADLE bound versus log concentration, the IC_{50} s for control and pretreated membranes respectively were 1.5 and 1.0 nM when DADLE was the displacing ligand and 35 and 75 nM for morphine sulphate, in good agreement with previous reports^{1,2,14}. However, it is unknown whether this reduction in the number of high-affinity binding sites is due to a loss of receptor sites or whether they are converted to sites of low affinity not detected in our binding assay. Recently, Bowen *et al.*¹⁵ have reported an interconversion of μ and δ receptors in rat striatal patches that is mediated by divalent cations and sulphydryl reagents. Because pretreatment of N4TG1 cells with DADLE results in a loss of 3 H-diprenorphine binding (a compound having similar affinity for μ and δ receptors) as well as 125 I-DADLE binding (data not shown), it seems unlikely that conversion from a δ to a μ form of receptor resulted in the observed down-regulation effect.

Numerous experiments *in vivo* and *in vitro* have indicated that enkephalin can mimic the biological effects of morphine, including the development of tolerance and physical dependence¹⁶. Previous experiments *in vivo* have led to the conclusion that neither the number nor the affinity of opiate receptors are altered after chronic treatment with morphine¹⁷⁻²⁰. We have confirmed this from measurements of the binding activities of three subtypes of opiate receptors including morphine (μ), enkephalin (δ) and benzomorphan binding sites²¹ after chronic morphine treatment. Thus, the effect of reducing receptor numbers may be selective for opioid peptides and may differ from the phenomena of *in vivo* morphine tolerance and dependence. However, the recent demonstration of the lack of cross-tolerance between a δ -agonist (DADLE) and a μ -agonist (sulphen-tanyl) in mouse vas deferens after chronic *in vivo* treatment with either ligand²² suggests that there may exist tolerance selective for a receptor subtype. N4TG1 cells contain δ -receptors only. The present studies demonstrate that only opioid peptides induce a decrease in enkephalin receptors; morphine (μ -agonist), ketocyclazocine (κ -agonist) and *N*-allylnormetazocine (σ -agonist) have no such effect, in fact they antagonize the effects of opioid peptides. These observations resemble the studies on mouse vas deferens²². Selective

Table 1 Specificity of the ligand-induced decrease in enkephalin receptors

Ligand	Concentration (M)	125 I-DADLE binding (% of control $\pm$ s.e.m.) (n)
		Ligand alone +
Agonists		
DADLE	10^{-7}	19.0 $\pm$ 3.2 (8)
	10^{-8}	27.6 $\pm$ 3.5 (8)
Met-Enkephalin	10^{-6}	18.4 $\pm$ 2.6 (3)
Leu-Enkephalin	10^{-6}	17.3 $\pm$ 2.2 (3)
[D-Ala ² ,NMePhe ⁴ ,Met(O) ⁵]enkephalin	10^{-6}	23.9 $\pm$ 4.6 (3)
[D-Ala ² ,Met ⁵]enkephalinamide	10^{-7}	20.1 $\pm$ 0.7 (3)
β -Endorphin	10^{-7}	42.1 $\pm$ 8.6 (5)
Ketocyclazocine	10^{-5}	99.0 $\pm$ 7.9 (3)
		55.8 $\pm$ 2.8* (2)
Morphine sulphate	10^{-5}	83.7 $\pm$ 8.2 (3)
	5×10^{-5}	72.0 $\pm$ 4.5 (4)
		27.9 $\pm$ 11.0* (3)
		40.9 $\pm$ 12.1* (3)
		82.8 $\pm$ 4.7† (3)
<i>N</i> -Allylnormetazocine	10^{-5}	71.0 $\pm$ 13.0 (2)
		89.3 $\pm$ 9.7† (3)
Antagonists		
Naloxone	10^{-5}	91.3 $\pm$ 34.7 (2)
Diprenorphine	10^{-7}	77.3 $\pm$ 16.1 (3)
Nalorphine	10^{-5}	81.8 $\pm$ 19.3 (3)
		96.7 $\pm$ 19.1* (3)
		72.5 $\pm$ 13.8† (3)
		96.5 $\pm$ 2.6* (2)

All ligands were preincubated with cells for 4 h before collection of the cells and preparation of membranes. As determined by Student's *t*-test, all values for peptides and morphine at 5×10^{-5} M differ significantly from the control (100%) ($P < 0.005$); all values for opiate agonists and antagonists except morphine at 5×10^{-5} M do not differ significantly from the control ($P > 0.1$). The values for opiates plus DADLE, except morphine (10^{-5} M) plus DADLE (10^{-7} M), differ significantly from the values for DADLE alone ($P < 0.005$).

* [DADLE] = 10^{-7} M.

† [DADLE] = 10^{-8} M.

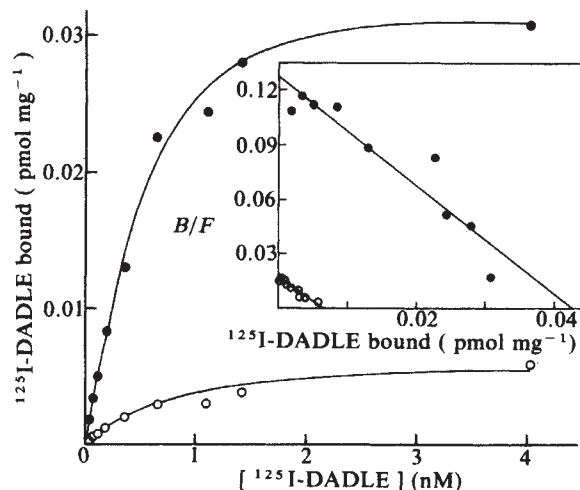


Fig. 3 Binding of ^{125}I -DADLE to N4TG1 membranes before and after preincubation of cells with unlabelled DADLE. Confluent cultures of N4TG1 cells were incubated with (○) or without (●) 10^{-8} M unlabelled DADLE and membranes were prepared as described in Fig. 1 legend. Aliquots of the membranes were incubated with various concentrations of ^{125}I -DADLE for 1 h, after which time bound ligand was determined by rapid filtration through glass fibre filters. Nonspecific binding was determined in the presence of 10^{-5} M unlabelled DADLE. Inset, Scatchard plot of the data. The control membranes had a K_d of 0.76 nM and B_{max} of 42.8 fmol mg^{-1} , while the respective values for the treated membranes were 0.83 nM and 6.4 fmol mg^{-1} .

desensitization due to the loss of opiate receptors could occur for δ -receptors after chronic opioid peptide treatment.

There are many possible mechanisms for the ligand-induced loss of receptors reported here. For example, desensitized low-affinity receptors may be induced by peptide which can no longer be detected by the present binding method. It is also possible that the receptors are cryptic after treatment with opioid peptides. The formation of (essentially) irreversible receptor-ligand complexes may also occur during the pretreatment. An understanding of the mechanism of receptor reduction may prove important in elucidating the physiological and pathological actions of opioids.

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Related amino acid sequences in neurofilaments and non-neuronal intermediate filaments

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The major constituents of mammalian neurofilaments are the three triplet proteins of molecular weight 200,000 (200K), 145K and 68K, which also co-migrate in slow axonal transport¹⁻⁵. Partial amino acid sequence data⁶ and chemical cleavage patterns^{7,8} indicate that various cell-specific non-neuronal intermediate filament proteins having molecular weights between 40,000 and 70,000 are structurally related, consistent with a common filament morphology. Here we extend this principle to the neurofilament 68K triplet protein, NF68. We show that NF68, muscle-specific desmin (52K) and mesenchymally derived vimentin (55K) are related proteins. Direct amino acid sequence analysis of a uniquely positioned marker peptide (5K) shows that in this region, NF68 shows ~42% sequence identity with vimentin and desmin, which have ~70% identity. These results are discussed with respect to neurofilament organization.

Treatment of porcine vimentin (55K) and desmin (52K) with the cysteine-specific reagent 2-nitro-5-thiocyanobenzoic acid (NTCB)⁹ yields two fragments (I and II)⁶. Whereas fragments I (37K) spanning the amino-terminal part of the two polypeptides were not further explored, the two carboxy-terminal fragments II (15K) were fully characterized and revealed 64% amino acid sequence identity between vimentin and desmin⁶. Similar treatment of NF68 isolated from porcine spinal cord¹⁰ gave rise to two fragments of 37K (I) and 28K (II) (Fig. 1). End-group analysis localized fragment II to the carboxy-terminus (Fig. 2). For direct sequence comparison, we examined cleavage at another amino acid residue, which is rare in intermediate filament proteins^{6-8,11,12}. BNPS-skatole, assumed to

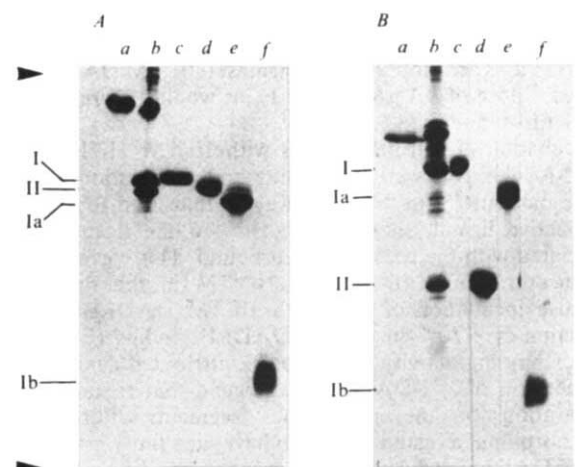


Fig. 1 SDS-polyacrylamide gel electrophoresis of vimentin, desmin and NF68 and their fragments. A and B show NF68 and vimentin, respectively; desmin behaved similarly to vimentin. Porcine proteins (a) were isolated as described elsewhere¹⁰⁻¹² and cleaved at cysteine^{6,9} to yield fragments I and II (b; see also Fig. 2). The two fragments were isolated by preparative gel electrophoresis⁶ (c and d) and fragments I (c) were further cleaved with BNPS-skatole¹³ to yield two fragments Ia and Ib, which were isolated by preparative gel electrophoresis⁶ (e, f). The purified Ib fragments (f) were freed of SDS and processed for protein sequencing as described elsewhere^{6,12,26}. Alternatively, peptides resulting from cysteine cleavage were mixed and subjected to tryptophan cleavage. Again Ib fragments were isolated from 20% polyacrylamide gels. For localization of fragments see Fig. 2.

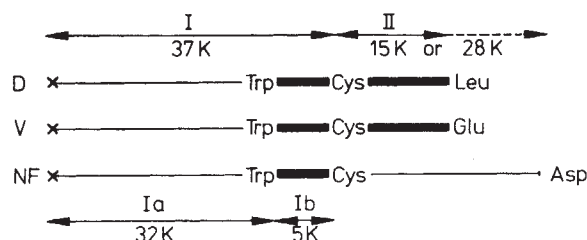


Fig. 2 Organization of the polypeptide chains of porcine desmin (D), vimentin (V) and the neurofilament 68K protein (NF). The relative positions of fragments I and II, obtained by cysteine cleavage of desmin and vimentin, have been given elsewhere⁶. In both cases fragments Ib obtained by tryptophan cleavage occupy the carboxy-terminal part of fragment I, as it has the same carboxy-terminal residue (0.8 mol threonine per mol polypeptide by carboxypeptidase Y digestion), whereas fragments Ia contain the blocked (X) amino-terminal end. NF68 fragment II must occupy the carboxy-terminus, as it has the same carboxy-terminal end-group as NF68 (0.5 mol aspartic acid per mol polypeptide determined by hydrazinolysis), whereas fragment I contains the amino-terminal blocking group and a new carboxy-terminal end (0.3 mol alanine per mol determined by hydrazinolysis). Fragment Ib is assigned again by the end-group results of fragments I, Ia and Ib. Amino-terminal fragments I have approximately the same molecular weights (see Fig. 1), whereas the carboxy-terminal fragments II differ between NF68 and the two other proteins and account for the marked molecular weight differences between NF68 (68K) and vimentin or desmin (55–52K). (For a comparison of the sequences of fragment II from desmin and vimentin, see ref. 6.) Portions of the polypeptides which have been sequenced are indicated by solid boxes.

cleave preferentially at tryptophan¹³, gave a fragmentation pattern indicating a single tryptophan residue situated ~40 residues amino-terminal from the sole cysteine residue documented previously⁶ (Figs 1, 2). Thus double chemical cleavage, first with NTCB and then with BNPS-skatole, provided two new fragments for all three proteins: a large amino-terminal fragment Ia (32K) and a small fragment Ib (5K) in addition to fragment II. Figure 2 indicates that the increased molecular weight of NF68 compared with desmin and vimentin (52–55K) may be accounted for by a larger carboxy-terminal fragment II, the sequence of which has not yet been determined.

The small size of fragments Ib allowed an assessment of the sequence homology suggested by the similar relative localization of tryptophan and cysteine residues in the three distinct proteins (Figs 2, 3). Although of different length (37 and 42 residues), the three fragments are related (Fig. 3). In the case of the vimentin and desmin sequences, 28 out of 39 residues

(72%) are identical; this per cent identity is comparable to that found previously for the 140 residues of the corresponding fragments II (64%)⁶. In the case of NF68 fragment Ib, the sequence homology is clearly lower, both in comparison with vimentin (43%) and with desmin (41%) fragments Ib. Thus the similarity in the relative location of cysteine and tryptophan in NF68 compared with vimentin and desmin (Fig. 2) is supported by a similarity in sequence of the resulting fragments Ib (Fig. 3). Although there is a small insertion in this NF68 fragment, many of the other amino acid exchanges are conservative. These results support the hypothesis that neuronal and non-neuronal intermediate filament proteins are related in sequence and belong to a multi-gene family, which is expressed according to certain rules of embryonic differentiation, and which largely coincide with histologically distinct cell types^{14–17}.

Present models of intermediate filament structure developed for epidermal keratins⁷ and extended to vimentin and desmin⁸ assume a similar 3-polypeptide unit, which contains regions of coiled-coil α -helix interspersed with regions of non- α -helix. Two long α -helices are thought to be present in the amino-terminal two-thirds of the molecules. As it is reasonable to assume that such α -helices have conserved sequences^{7,8} and because our sequence data so far cover only that part of the molecule assumed to be non-helical, more extensive protein data may reveal a higher degree of sequence similarity between different intermediate filament proteins than is presently indicated (our results and ref. 6). Note, however, that in the case of NF68, chain length variation seems to be achieved by an alteration, insertion or addition in the carboxy-terminal domain (Figs 1, 2), whereas previous studies on epidermal keratins indicated that the region between the two helical domains is the major determinant of variant molecular size⁸.

The sequence homology observed between NF68 and two distinct non-neuronal intermediate filament proteins agrees with our finding that purified 68K protein alone can self-assemble *in vitro* to form smooth 10-nm filaments¹⁰. What then is the function of the two higher molecular weight neurofilament triplet proteins? They may either act as associated proteins binding to a filament backbone provided solely by the 68K protein, as indicated by antibody decoration studies for the 200K protein present in native neurofilaments^{4,18}, or may themselves form part of the filament backbone. The latter possibility is suggested but not proved by several observations. Although the three triplet proteins possess unique and distinct antigenic determinants^{4,19}, several studies suggest the presence of common determinants^{2,4,17,19–21} defined by a recently characterized hybridoma antibody, which not only recognizes the three triplet proteins but also at least several distinct non-neuronal

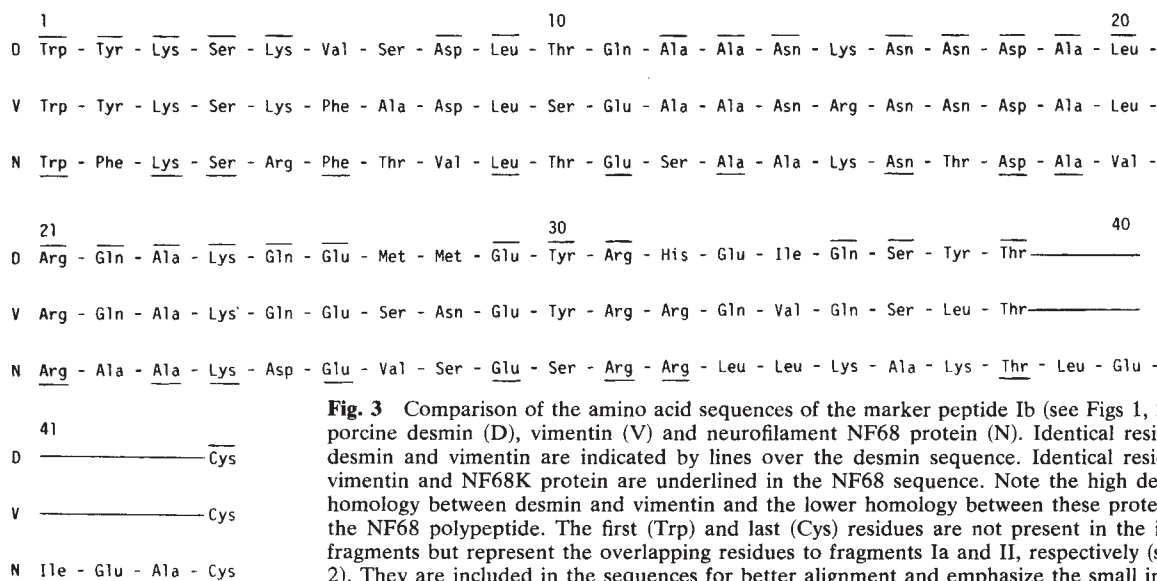


Fig. 3 Comparison of the amino acid sequences of the marker peptide Ib (see Figs 1, 2) from porcine desmin (D), vimentin (V) and neurofilament NF68 protein (N). Identical residues in desmin and vimentin are indicated by lines over the desmin sequence. Identical residues in vimentin and NF68K protein are underlined in the NF68 sequence. Note the high degree of homology between desmin and vimentin and the lower homology between these proteins and the NF68 polypeptide. The first (Trp) and last (Cys) residues are not present in the isolated fragments but represent the overlapping residues to fragments Ia and II, respectively (see Fig. 2). They are included in the sequences for better alignment and emphasize the small insertion in the carboxy-terminal region of the NF68 fragment Ib.

intermediate filament proteins²². If these results indicate common functional amino acid sequences, the two higher molecular weight triplet proteins may be incorporated at least in part into the filament backbone. This possibility is also suggested by their relative abundance compared with the 68K protein^{1-5,10,17-21,23}, which makes it difficult to envision them solely as associated proteins in the sense of microtubule-associated proteins present in microtubules²⁴. In addition, in the invertebrate *Myxicola*, neurofilaments have no NF68 and consist of only two polypeptides²⁵ having molecular weights of 150K and 160K, and which are antigenically related to mammalian intermediate filament proteins^{17,22}. Clearly more chemical and structural information is needed to elucidate neurofilament architecture.

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Note added in proof: We have recently found that bovine glial fibrillary acidic protein (GFA) shows the same distribution of tryptophan and cysteine residues as given in Fig. 2 for desmin and vimentin.

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Influence of ionic current on Na⁺ channel gating in crayfish giant axon

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In studies of nerve excitation it is important to examine the possible interaction of voltage-sensitive gating mechanisms with components forming the pathway of ionic channels. There is evidence that permeant ions and gating are independent in Na⁺ channels^{1,2} of nerve fibres, but not in K⁺ channels³. Of interest in this and other studies of excitability is the determination of the physical basis for the resistance in series with the axon membrane (R_s)⁴. In an attempt to determine whether an appreciable R_s is associated with individual Na⁺ channels⁵, we have compared gating kinetics in conditions in which the ionic current through individual channels is varied while the total membrane current remains constant. The number of conducting Na⁺ channels, and thus their contribution to R_s , was lowered

by addition of tetrodotoxin⁶. We report here that no more than 10% of the resistance in 'series' with activation gates is associated with the Na⁺ channel, and give some preliminary estimates for minimum distances between gates and ion-binding sites within the Na⁺ channel.

The most common method of measuring R_s uses the response to a hyperpolarizing step in current-clamp, a condition in which most Na⁺ channels are closed. The influence of R_s is seen most clearly, however, in a dependence of Na⁺ channel gating kinetics on the magnitude of Na⁺ currents. As we wished to make no assumptions regarding the identity of these two resistances, our experiments used very small ionic currents and no electronic compensation for R_s . Crayfish giant axons were voltage-clamped and internally perfused^{7,8}. Two external solutions were required: 100% Na contained (mM) Na⁺, 210.2; K⁺, 1; Mg²⁺, 2.6; Ca²⁺, 13.5; Cl⁻, 293.3; HCO₃⁻, 2.3; HEPES, 2.5; 4-aminopyridine, 2, pH 7.70 ± 0.05 at 8 °C; 6% Na was identical except that all but 13.8 mM of Na⁺ was replaced by tetramethylammonium ion. The difference in liquid junction potential at the reference electrode on switching between these solutions was <1 mV and has been ignored. Measurements were made on the kinetics of closing of activation and inactivation gates, first in 6% Na, then in 100% Na-3.2 nM tetrodotoxin (TTX). Pulse protocols were then repeated in each solution with 100 nM TTX. Conditions were thus set so that closing kinetics could be measured either with many channels open, but low ionic current per channel, or with few channels open but high current per channel. The experiments were designed to keep total ionic current small and equal in the two cases. After several preliminary experiments to establish the best procedures, the following data were obtained from five axons.

Families of Na⁺ currents used in the determination of inactivation time constants (τ_h) are shown in Fig. 1; τ_h is a measure of the rate of decay of Na⁺ currents under a maintained depolarization. 100% Na+3.2 nM TTX provided a good match of peak currents with those in 6% Na, particularly at lower depolarizations, which were of primary interest. Figure 2 plots τ_h against V_m ; relatively little variation is seen among the different fibres in each solution. The shift in the points from 6% Na (open symbols) to 100% Na+3.2 nM TTX (solid symbols), measured in the region of greatest slope (-20 to -40 mV),

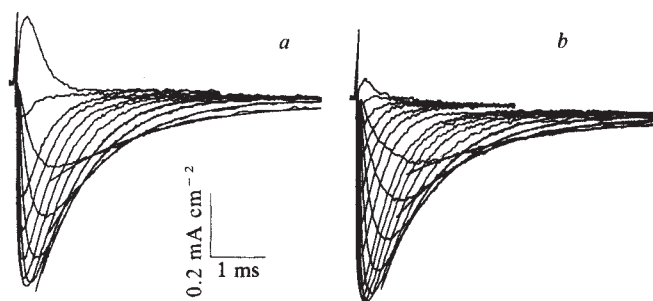


Fig. 1 Families of Na⁺ currents. *a*, 6% Na; *b*, 100% Na+3.2 nM TTX. Three consecutive sweeps were averaged by the computer in all cases. K⁺ currents were reduced by 4-aminopyridine^{15,16} and eliminated by subtraction of records in 100 nM TTX. Pulses at low depolarizations were of longer duration than shown to allow an accurate determination of exponential fits to Na⁺ current decay. Pulses at high depolarizations were of short duration. Fits of single exponentials to inactivation were made using strict and consistent criteria: there was no adjustment of the baseline and the fit was made between 10 and 70% of the peak current. Fits at the four lowest depolarizations are shown superimposed. It has been previously demonstrated^{14,17} that inactivation kinetics in the crayfish axon are well described by a single time constant. Holding potential, -76 mV; 50 ms prepulses to -116 mV and a 0.5 ms return to -76 mV preceded depolarizations to *a* (6% Na): -36, -31, -26, -21, -16, -11, -6, -1, +4, +14, and +24 mV. In 100% Na+3.2 nM TTX (*b*), additional depolarizations to +34, +44, +54, +64 and +74 mV are shown. External solutions are described in the text. The internal perfusate contained (mM) K⁺, 230; Na⁺, 5; F⁻, 119; Cl⁻, 5; citrate ion, 37; mannitol, 93; pH 7.35.

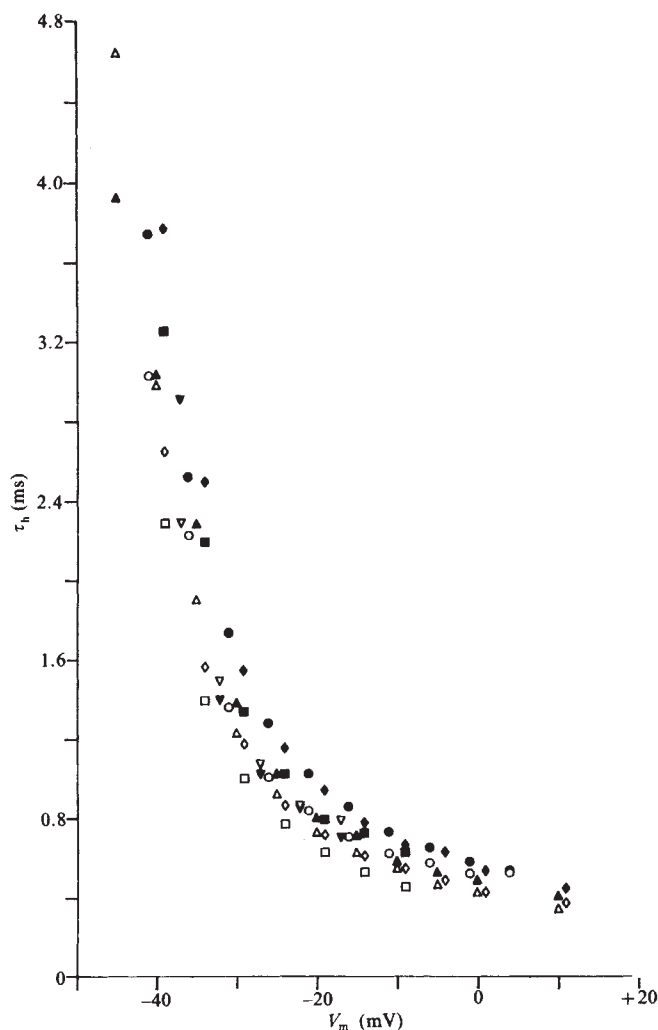


Fig. 2 Inactivation time constants plotted against membrane potential. Data from five axons. Open symbols, 6 % Na; solid symbols, 100 % Na + 3.2 nM TTX. Single exponentials were fitted to Na⁺ current decays using a weighted least-squares procedure.

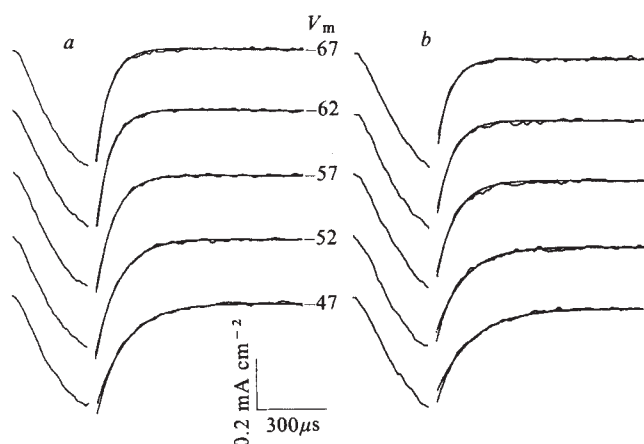


Fig. 3 Na⁺ tail currents. *a*, 6 % Na; *b*, 100 % Na + 3.2 nM TTX. Three consecutive sweeps were averaged. Holding potential, -77 mV. A hyperpolarizing prepulse to -117 mV removed resting inactivation. The fibre was depolarized to -22 mV for 0.4 ms and V_m was then changed to the values shown. Records of ionic current are shown after subtraction of traces in 100 nM TTX. Exponential fits are superimposed on tail currents and are generally adequate. At the highest depolarizations used (~ -45 mV), a second small (<10%) and very slow component ($\tau \approx \tau_h$) was sometimes seen and was subtracted before fitting. Only 4 of the 44 points used in calculating shifts in Fig. 4 required this procedure.

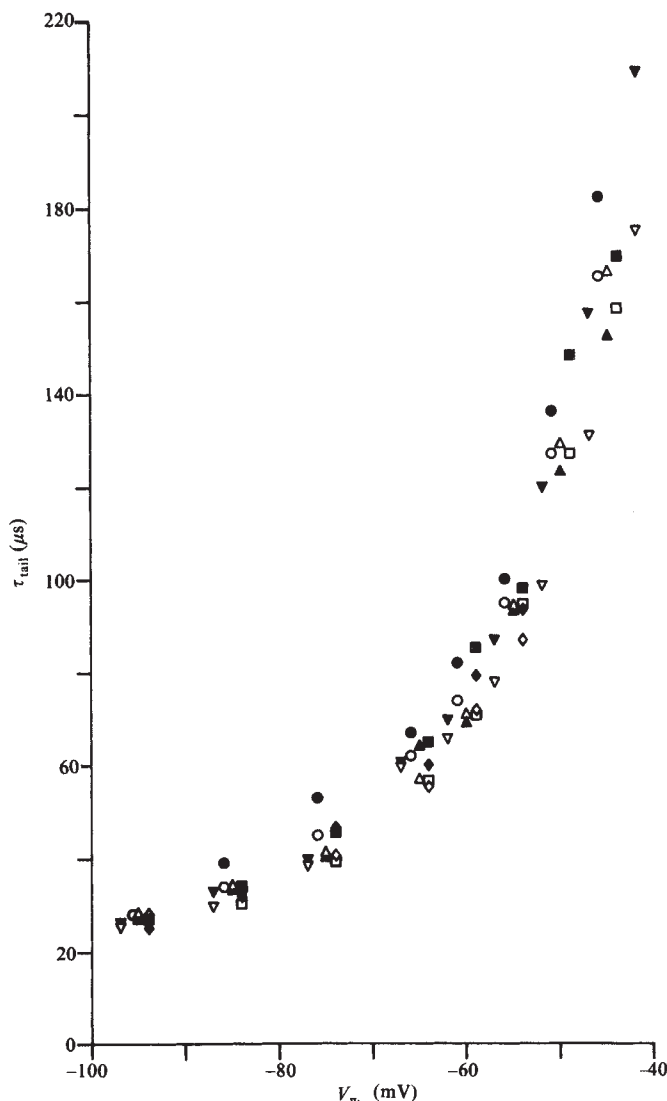


Fig. 4 Na⁺ tail current time constants plotted against membrane potential. Points are from the same axons as in Fig. 2. Open symbols, 6 % Na; solid symbols, 100 % Na + 3.2 nM TTX.

is $+3.1 \pm 1.0$ mV ($\pm$ s.e.m.). This shift is small and is in the opposite direction to that expected for a series resistance. For inward currents, the IR_s voltage drop causes measured V_m values to be more negative than actual membrane potentials.

An example of Na⁺ tail currents is given in Fig. 3. In contrast to τ_h , τ_{tail} measures the rate of decay of Na⁺ conductance when a depolarization is abruptly interrupted and V_m returned to a more negative value⁹. Fits to single exponentials are superimposed and are seen to be generally satisfactory. Figure 4 shows τ_{tail} plotted against V_m . The shift along the voltage axis from 6 % Na (open symbols) to 100 % Na + 3.2 nM TTX (solid symbols), measured between -70 and -45 mV, is -2.0 ± 0.4 mV. This is again small, but this time is consistent with a channel-associated R_s .

Current-clamp techniques give a total R_s of 4–6 $\Omega\text{-cm}^2$ in crayfish axons⁷. Furthermore, with no R_s compensation, voltage shifts in τ_h - V_m curves on changing from 100 % Na (no TTX) to 6 % Na, divided by the difference in current amplitude, suggest an R_s of 6–7 $\Omega\text{-cm}^2$. We shall use 6 $\Omega\text{-cm}^2$ as a reasonable value in order to estimate the magnitude of some possible sources of error. At midpoints of the voltage ranges used to determine shifts (-30 mV for τ_h and -58 mV for τ_{tail}), average maximum currents were 0.38 mA cm^{-2} (τ_h) and 0.41 mA cm^{-2} (τ_{tail}), suggesting that errors due to uncompensated Schwann cell R_s should be at most ~ 2.4 mV. This is confirmed by the observation that despite the significant ranges of maximum currents in different axons in the same solution (for example,

0.2–0.5 mA cm⁻² used to measure τ_h in 6 % Na; 0.3–0.6 mA cm⁻² in the case of τ_{tail} in 6 % Na), the time constants vary over only a 2-mV span at these potentials. Our experiments have been designed to reduce such errors further by keeping current magnitudes equal in the two external solutions. The mean difference (value in 6 % Na minus that in 100 % Na + 3.2 nM TTX) was 0.06 mA cm⁻² for measuring τ_h and 0.20 mA cm⁻² for τ_{tail} . $R_s \Delta I$ then represents a small correction so that the shifts along the voltage axis in changing from 6 % Na to 100 % Na + 3.2 nM TTX become $+2.7 \pm 1.0$ mV for τ_h and -3.2 ± 0.4 mV for τ_{tail} .

We calculate that in the five axons analysed for shifts, the difference in peak Na⁺ currents between 100 % Na (no TTX) and 6 % Na was ~ 5 mA cm⁻². Since the Na⁺ current per channel should be unchanged by the addition of 3.2 nM TTX¹⁰, the shift in gating kinetics expected if all the R_s were associated with individual Na⁺ channels is ~ 30 mV. Our results suggest that with regard to activation, almost 90% of the R_s is not of this form and is probably a reflection of the Schwann cell connective tissue sheath. Furthermore the small residual resistance shows no systematic voltage dependence in the range of V_m in which activation is highly voltage-dependent. Mathias *et al.*

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Nerve impulses increase the phosphorylation state of protein I in rabbit superior cervical ganglion

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Protein I, a neurone-specific protein concentrated at synapses, is present in most, and probably all, presynaptic nerve terminals where it is at least partially associated with neurotransmitter vesicles (refs 1–5 and P. De Camilli, W. B. Huttner, R. Cameron, M. Harris and P.G., in preparation). Protein I is a major endogenous substrate in nervous tissue for both cyclic AMP-dependent¹ and calcium/calmodulin-dependent^{6–8} protein kinases. Neurotransmitters^{9,10}, apparently acting through cyclic AMP, and depolarizing agents^{9–11}, apparently acting through calcium, have been shown to increase the state of phosphorylation of protein I in intact preparations of the central and peripheral nervous systems. To determine whether impulse conduction regulates the state of phosphorylation of protein I, we have now studied this process in the rabbit superior cervical ganglion, a well characterized neuronal preparation that is suitable for both physiological and biochemical studies. Using a technique developed to quantify the state of phosphorylation of the small amounts of protein I contained in ganglion tissue, we show here that in physiological conditions brief periods of impulse conduction increase the state of phosphorylation of protein I in this ganglion.

New Zealand White rabbits (1.5–2.5 kg) were used in this study. The two superior cervical ganglia of each rabbit contained almost identical amounts of dephospho-protein I when neither ganglion was stimulated (Fig. 1a). Therefore, one ganglion from each rabbit served as the 'test' ganglion and the contralateral ganglion as the control. When the preganglionic nerves supplying the test ganglia were stimulated at 10 Hz for 30 s (a frequency of impulse conduction observed *in vivo*¹²), there was a decrease in the amount of dephospho-protein I compared with that in the contralateral control ganglion (Fig. 1b). As the

*al.*⁵ have postulated that a channel-associated, voltage-dependent R_s might account for gating charge movement; our results suggest that this is inappropriate for nerve Na⁺ channels. Salzberg *et al.*¹¹, using an optical probe, measured the Schwann cell contribution to R_s in squid axons as 2.8 Ω -cm².

The shifts that were measured in our experiments are small and are in opposite directions. One possible explanation might reside in an electrostatic interaction in which a Na⁺ ion bound at a site within the channel^{12,13} stabilizes the voltage-sensitive components of gates in their open states¹⁴. Because of the opposite voltage dependence of τ_h and τ_{tail} in the V_m ranges considered, a slowing of each time constant would result in shifts of opposite sign. From the K_d of 368 mM measured by Hille¹², and the Na⁺ concentration in 100 % Na, at least one site in the channel might be occupied for about one-third of the time the channel is open. Then, for a shift of 3 mV, and assuming a uniform dielectric with no intervening counterions, we calculate the minimum distance between Na⁺ ion and gate voltage sensor to be 20 Å. If the effective dielectric constant is less than 81, this value would be proportionally higher.

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total amount of protein I, determined by radioimmunoassay, was not altered by nerve stimulation (data not shown), the results demonstrate that in physiological conditions impulse conduction increased the state of phosphorylation of protein I in the rabbit superior cervical ganglion. In contrast to stimulation of preganglionic nerves (orthodromic impulse conduction), stimulation of postganglionic nerves (antidromic impulse conduction) at 10 Hz for 30 s did not alter protein I phosphorylation (Fig. 1c). Furthermore, the effect of preganglionic nerve stimulation on protein I phosphorylation was dependent on extracellular calcium (compare Fig. 1b with d).

Tables 1 and 2 show the state of phosphorylation of protein I in the rabbit superior cervical ganglion as a function of the number of impulses conducted along the preganglionic nerve. In one series of experiments (Table 1a), the preganglionic nerves supplying test ganglia were stimulated at 10 Hz for various periods of time and the amount of dephospho-protein I present immediately after the period of nerve stimulation was determined. An increase in protein I phosphorylation was first observed after 10 s of nerve stimulation (100 impulses) and appeared to be maximal after 20 s (200 impulses). In a related series of experiments (Table 1b), the preganglionic nerves supplying test ganglia were stimulated at 10 Hz for various periods of time as in Table 1a, and the amount of dephospho-protein I present 30 s after the initiation of nerve stimulation was determined. In these conditions, 2 s of nerve stimulation (20 impulses) significantly increased protein I phosphorylation and 5 s of nerve stimulation (50 impulses) appeared to produce a maximum in protein I phosphorylation.

In further experiments (Table 2), the preganglionic nerves supplying test ganglia were stimulated for 5 s at various frequencies and the amount of dephospho-protein I present 30 s after the initiation of nerve stimulation was determined. The state of phosphorylation of protein I was increased when ganglia were stimulated at 5 Hz (25 impulses) and 10 Hz (50 impulses), but not at lower frequencies. Figure 2 shows the change in dephospho-protein I observed in response to 5 s of preganglionic nerve stimulation at 10 Hz as a function of the time after initiation of nerve stimulation. An increase in the state of phosphorylation of protein I was first observed 20 s after initiation of nerve stimulation and appeared to be maximal at

Fig. 1 Regulation by impulse conduction of the state of phosphorylation of protein I in the rabbit superior cervical ganglion. Rabbits were injected intravenously with 1 ml (50 mg) sodium pentobarbital (Nembutal, Abbott) and then were killed by air embolism. The two superior cervical ganglia of each rabbit, with their pre- and postganglionic nerves, were quickly dissected, placed in a chamber and superfused at room temperature with oxygenated Locke's buffer (mM): 136 NaCl; 5.6 KCl; 20 NaHCO₃; 1.2 NaH₂PO₄; 2.2 CaCl₂; 1.2 MgCl₂; 0.18% glucose (pH 7.4). The ganglia were carefully decapsulated and then superfused for 30–45 min with: *a*–*c*, standard Locke's buffer; *d*, calcium-free Locke's buffer (2.2 mM MgCl₂ and 1 mM EGTA were substituted for 2.2 mM CaCl₂). One ganglion from each rabbit served as the 'test' ganglion, and the other as the control. *a*, Test ganglia were mounted in suction electrodes, but not stimulated. *b*, *d*, The preganglionic nerve supplying the test ganglion was stimulated via a suction electrode with supramaximal pulses at 10 Hz for 30 s. The effectiveness of stimulation was monitored by recording compound action potentials from postganglionic nerves using a second suction electrode. *c*, The postganglionic nerves supplying the test ganglion were stimulated at 10 Hz for 30 s using a suction electrode. Immediately after the 30 s period of nerve stimulation, test and contralateral control ganglia were homogenized in 400 µl of 1% SDS using conical glass tissue homogenizers (Belco). The SDS homogenates were boiled for 5 min and then centrifuged for 2 min in a Beckman microfuge. The supernatants ('SDS extracts') contained essentially all the total protein I as determined by radioimmunolabelling of gels⁵. SDS extracts were used to quantify the amount of dephospho-protein I and the total amount of protein I. The former was assayed by a 'back phosphorylation' technique in which the protein I in SDS extracts was first immunoprecipitated and then phosphorylated with purified protein kinase. The immunoprecipitation reaction was carried out at 0–4 °C in plastic microfuge tubes. Duplicate aliquots (60 µl) of the SDS extract from each ganglion were adjusted by addition of a 200 µl solution to contain (mM; final concentrations): 250 NaCl; 50 NaF; 14 EDTA; 10 sodium phosphate, pH 7.4; 1.5% (v/v) Nonidet P-40. To these were added 4 µl of the γ-globulin fraction of anti-protein I rabbit antiserum. The mixture was incubated for 20 min; then 3 µg of purified rabbit IgG (Sigma) and 20 µl of the IgG fraction of goat anti-rabbit IgG (Sigma; 1 ml of goat IgG precipitated 2–3 mg of rabbit IgG) were added. After incubation for 3 h, the mixture was centrifuged in a microfuge for 1 min. The pellet was washed with 200 µl ice-cold 150 mM NaCl/10 mM sodium phosphate, pH 7.4, and then dissolved in 50 µl of ice-cold 11 mM citric acid (final pH ~3). The dissolved pellet was phosphorylated as described elsewhere¹⁰, with the following modifications. The assay mixture (final volume, 70 µl) contained: 50 mM HEPES, pH 7.4; 10 mM MgCl₂; 0.1 µM purified catalytic subunit (beef heart cyclic AMP-dependent protein kinase, provided by A. C. Nairn); 15 mM dithiothreitol; 0.005% Nonidet P-40. The phosphorylation reaction was initiated by adding 30 µl [³²P]ATP (final concentration 2–3 µM, specific activity 0.5–1 × 10⁶ c.p.m. nmol⁻¹), and was carried out at 30 °C for 30 min. The reaction was terminated by boiling the mixture in 'SDS-stop solution' as described elsewhere¹⁰. The boiled phosphorylated extract was then subjected to SDS-polyacrylamide gel electrophoresis as described previously¹⁰, except that the lower (resolving) gel contained 9% acrylamide with a ratio of acrylamide to methylenebisacrylamide of 30:1.6. The ³²P contained in the protein I region of the dried gels was located by autoradiography and quantified using Cerenkov radiation. In the conditions used, phosphorylation of protein I was maximal, and was proportional to the amount of extract over a 10-fold concentration range of ganglion extract. (Peptide mapping analysis of protein I in SDS extracts from control ganglia revealed that 0.78 ± 0.03 (mean ± s.e.m. of 20 ganglia, 8 experiments) mol of phosphate was incorporated per mol of protein I on the serine residue of peptide I (ref. 20), the region of protein I that is phosphorylated both by cyclic AMP-dependent and calcium-dependent protein kinases.) The amount of dephospho-protein I in test ganglia was compared with that in contralateral control ganglia. Values shown are mean ± s.e.m. The numbers of pairs of ganglia used were: *a*, 6; *b*, 7; *c*, 6; *d*, 6. The total amount of protein I in the SDS extract from each ganglion was determined in triplicate by a detergent-based radioimmunoassay for protein I (ref. 21). None of the experimental procedures used here altered significantly the total protein I content of test ganglia compared with that of contralateral control ganglia. Thus, changes observed in the amount of dephospho-protein I on impulse conduction reflected changes in the state of phosphorylation of protein I and not changes in the total amount of protein I in the ganglion. * Significantly different from control (*P* < 0.05) by two-tailed *t*-test.

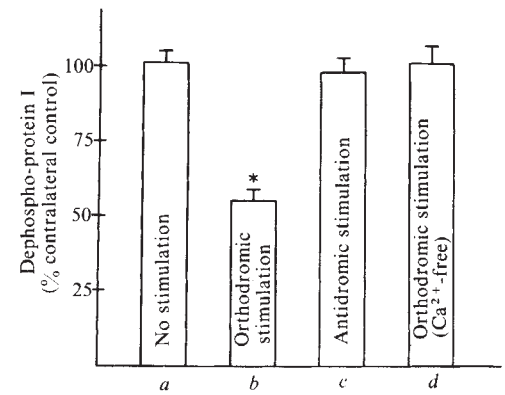


Table 1 Effect of number of impulses of constant frequency (10 Hz) on protein I phosphorylation

<i>a</i>	No. of impulses	Duration of nerve stimulation (s)	Dephospho-protein I immediately after nerve stimulation (% contralateral control)	<i>b</i>	No. of impulses	Duration of nerve stimulation (s)	Dephospho-protein I 30 s after initiation of nerve stimulation (% contralateral control)
	0	0	102 ± 4 (6)		0	0	102 ± 4 (6)
	50	5	107 ± 4 (3)		10	1	91 ± 14 (3)
	100	10	69 ± 4 (3)*		20	2	72 ± 4 (3)*
	200	20	54 ± 3 (3)*		50	5	58 ± 8 (7)*
	300	30	56 ± 4 (7)*		300	30	56 ± 4 (7)*

The preganglionic nerve supplying one ganglion of each rabbit was stimulated via a suction electrode at 10 Hz for various periods of time. Stimulated and contralateral control ganglia were homogenized in 1% SDS *a*, immediately after nerve stimulation; and *b*, 30 s after the initiation of nerve stimulation. Dephospho-protein I was determined as described in Fig. 1 legend, and the amount in the stimulated ganglion was compared with that in the contralateral control ganglion. Data are expressed as mean ± s.e.m. Values in parentheses represent the number of pairs of ganglia.

* Significantly different from control (*P* < 0.05) by two-tailed *t*-test.

Table 2 Effect of number of impulses occurring during a constant interval (5 s) on protein I phosphorylation

No. of impulses	Frequency (Hz)	Dephospho-protein I (% contralateral control)
0	0	102 ± 4 (6)
5	1	101 ± 15 (3)
10	2	94 ± 20 (3)
25	5	74 ± 6 (5)*
50	10	58 ± 8 (7)*

The preganglionic nerve supplying one ganglion of each rabbit was stimulated via a suction electrode for 5 s at various frequencies. Stimulated and contralateral control ganglia were homogenized in 1% SDS 30 s after the initiation of nerve stimulation. Dephospho-protein I was determined as described in Fig. 1 legend, and the amount in the stimulated ganglion was compared with that in the contralateral control ganglion. Data are expressed as mean ± s.e.m. Values in parentheses represent the number of pairs of ganglia.

* Significantly different from control (*P* < 0.05) by two-tailed *t*-test.

30–60 s, after which the state of phosphorylation of protein I returned to control levels.

Related studies (E.J.N. and P.G., in preparation) on the rabbit superior cervical ganglion have indicated that the ganglion contains two 'pools' of protein I. One pool, representing ~60% of total ganglion protein I and which is located in presynaptic nerve terminals, disappears on surgical denervation of the ganglion but is unaffected by brief exposure to the protein synthesis inhibitor, cycloheximide. The other pool, representing ~40% of total ganglion protein I, is located in the cell bodies of the ganglionic neurones. This postsynaptic pool is unaffected by denervation but is decreased by brief exposure to cycloheximide. Thus, postsynaptic protein I appears to represent newly synthesized protein I, presumably en route to the nerve terminals arising from the ganglionic cell bodies.

The state of phosphorylation of protein I is increased not only by impulse conduction, but also by dopamine and by high K⁺ in intact rabbit superior cervical ganglia (E.J.N. and P.G., in preparation). In contrast, dopamine and high K⁺ did not alter protein I phosphorylation in denervated ganglia. Furthermore, the effect of impulse conduction on the state of

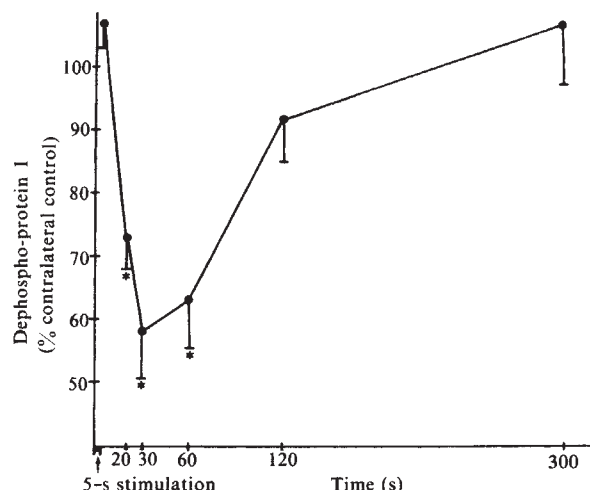


Fig. 2 Effect of a brief period (5 s) of impulse conduction on amount of dephospho-protein I, as a function of time after stimulation. The preganglionic nerve supplying one ganglion of each rabbit was stimulated via a suction electrode at 10 Hz for 5 s; stimulated and contralateral control ganglia were homogenized in 1% SDS at various times afterwards. Dephospho-protein I was determined as described in Fig. 1 legend, and the amount in the stimulated ganglion compared with that in the contralateral control ganglion. Values shown are mean $\pm$ s.e.m. The number of pairs of ganglia used ranged from 3 to 7. * Significantly different from control ($P < 0.05$) by two-tailed t -test.

phosphorylation of protein I was not blocked by those neurotransmitter antagonists that abolish the three postsynaptic potentials observed in the ganglion. These results suggest that the state of phosphorylation of presynaptic protein I, but not that of postsynaptic protein I, is regulated by impulse conduction, by dopamine, and by high K^+ .

We have shown that brief periods of impulse conduction at physiological frequencies¹² increase the state of phosphorylation of protein I in the rabbit superior cervical ganglion. This effect is dependent on extracellular calcium, suggesting that impulse conduction increases protein I phosphorylation through the activation of calcium-dependent protein kinase(s)⁸. We have also found that dopamine and 8-bromo-cyclic AMP increase the state of phosphorylation of protein I in rabbit ganglia, in the presence or absence of extracellular calcium (E.J.N. and P.G., in preparation), suggesting that these agents increase protein I phosphorylation through the activation of cyclic AMP-dependent protein kinase¹. It is interesting to note in this context that cyclic AMP^{13,14} and calcium^{15,16} have also been shown to modulate neurotransmitter release at various synapses. Catecholamines including dopamine, apparently acting through cyclic AMP¹⁷, and brief periods of impulse conduction, apparently acting through calcium^{18,19}, produce long-lasting facilitation of neurotransmitter release in vertebrate sympathetic ganglia. It is possible that an increase in the state of phosphorylation of protein I may be one of the mechanisms by which certain neurotransmitters and brief periods of impulse conduction facilitate neurotransmitter release.

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Use of HLA loss mutants to analyse the structure of the human major histocompatibility complex

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Class I loci of the human major histocompatibility complex (MHC) encode 44,000-molecular weight polypeptides that associate noncovalently with β -microglobulin¹. Included in this category are the *HLA-A*, *-B* and *-C* loci (Fig. 1a), which are extensively polymorphic². HLA-specific cDNA clones^{3,4} now allow this polymorphism to be studied at the DNA level. However, the lack of sufficient amino acid sequence data for all but a few of the class I antigens presents a major difficulty in the allelic assignment of those DNA clones obtained in man³⁻⁵ and mouse⁶⁻⁸. HLA loss variants produced with deletion-inducing ionizing radiation offer a means of partially circumventing the time-consuming and difficult task of sequencing HLA class I gene products for identifying restriction enzyme digest fragments of DNA and recombinant DNA clones. P.K. *et al.*⁹ used γ -ray irradiation followed by immunoselection to obtain many mutants that no longer expressed one or more HLA specificities from the human lymphoblastoid cell line LCL 721. Only the expression of *HLA-B8* was lost in one class of mutant, while expressions of *HLA-B8* and at least one additional *cis*-linked HLA allele were lost in mutants of the second class. Some mutants lost expression of the entire haplotype, that is, *HLA-A1*, *-B8* and *-DR3* (ref. 9) as well as *SB* (refs 4, 10 and unpublished results). Recently, anti-*HLA-B5* and anti-*HLA-A2* sera have been used to isolate mutants that have lost expressions of one or more loci of the haplotype on the other chromosome 6 of LCL 721 (Table 1)¹⁰. Data presented here illustrate how one cDNA clone of an HLA class I gene, pHLA-1, in combination with γ -ray-induced HLA loss variants, will be used to identify recombinant DNA HLA clones and to correlate individual DNA restriction fragments with expression of specific HLA alleles.

pHLA-1 (Fig. 1b, c) contains a 513-base pair (bp) cDNA insert which corresponds to the COOH-terminal 46 amino acids of an HLA class I heavy chain plus a portion of the 3'-noncoding region of the mRNA³. To make a preliminary estimate of HLA class I gene family size, genomic DNA was isolated from a heterozygous LCL, 721 (Table 1) and probed with pHLA-1. On hybridization, 721 genomic DNA digested with the restriction enzyme, *HindIII*, contained 12 distinct bands (Fig. 2a) which ranged in size from 29 to 1.7 kilobases (kb) and differed in intensity.

At least three factors contribute to the number of bands of genomic DNA that hybridize with pHLA-1 and thereby affect

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our estimate of the number of class I loci. First, despite the fact that the restriction enzyme used does not cut within the cloned pHLA sequence, it might cut at sites within intervening sequences that separate parts of the pHLA-1 sequence in genomic DNA. Thus, enzymatic treatment could yield two or more fragments derived from a single gene that hybridize with pHLA-1. Second, because the bands which hybridize pHLA-1 differ markedly in intensity, it is possible that the more intense bands represent multiple distinct genes or gene copies. Third, some class I genes may not hybridize with pHLA-1 although this seems unlikely as the amino acid sequence homology¹¹ (88%) between HLA-B7 and HLA-A2 for a significant portion of the heavy chain represented by the coding region of pHLA-1 suggests that the sequence of this region of HLA class I antigens is highly conserved.

Lack of information about the three points above prevents a direct determination of the number of class I genes in the human MHC from the genomic DNA hybridization pattern of pHLA-1. To what extent pHLA-1-positive *Hind*III bands of LCL 721 (Fig. 2a) represent class I loci in addition to *HLA-A*, *-B* and *-C*, for example, human analogues¹²⁻¹⁴ of mouse *TL* (ref. 15) and *Qa-2* (ref. 16), or multiple copies of *HLA-A*, *-B* and *-C* genes within a haplotype, will require analysis of the corresponding genomic clones. Furthermore, the lack of substantial amino acid sequence information for most *HLA* alleles makes the correlation of genomic clones with allelic specificities exceedingly difficult.

We investigated the usefulness of γ -ray-induced *HLA* loss mutants for identifying bands in Southern blots with specific *HLA* loci and their alleles by probing DNA from several such mutants of LCL 721 (Table 1) with pHLA-1. Figure 2 shows the banding pattern found after hybridization of *Hind*III-digested DNA from six mutants with nick-translated¹⁷ pHLA-1. Four of the mutants had banding patterns that differed from that of the parental LCL 721 cells by either a loss or gain of a band.

Mutant 721.4, a *HLA-B8* loss mutant (Table 1), has every band found in LCL 721 and a new 1.4-kb band not found in LCL 721 that could be explained by a deletion within a larger normal *Hind*III fragment. (Comparison of the digest of 721.4 with those of LCL 721 and 721.18 suggests that possible candidates for the larger band are the 23.5-kb and 1.7-kb bands. These interpretations can be tested by cloning the 1.4-kb fragment and using it to probe the other *Hind*III fragments.)

Simultaneous losses of expression of *HLA-A1*, *-B8* and *-DR3* are associated with a microscopically visible deletion on

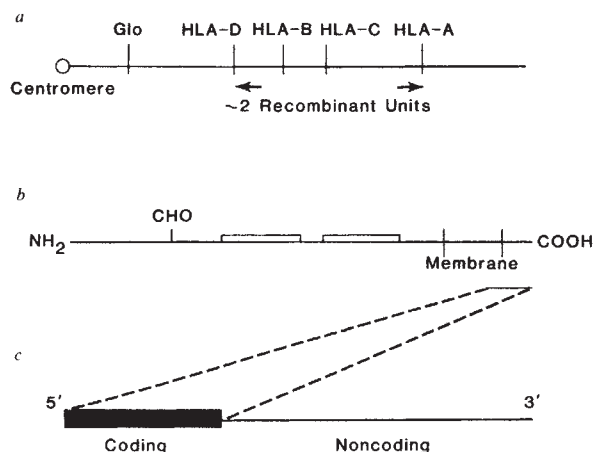


Fig. 1 a, A genetic map of the short arm of human chromosome 6. The genetic markers are: GLO, glyoxalase I. The major histocompatibility loci are *HLA-A*, *HLA-B*, *HLA-C* and *HLA-D*. b, A schematic depiction of an *HLA* class I antigen (*HLA-A*, *-B* or *-C*) heavy chain in the plasma membrane. CHO, the carbohydrate side chain and $\square$, disulphide loop. c, Diagram of the pHLA-1 513-bp cDNA insert and its relationship to a *HLA* class I heavy chain.

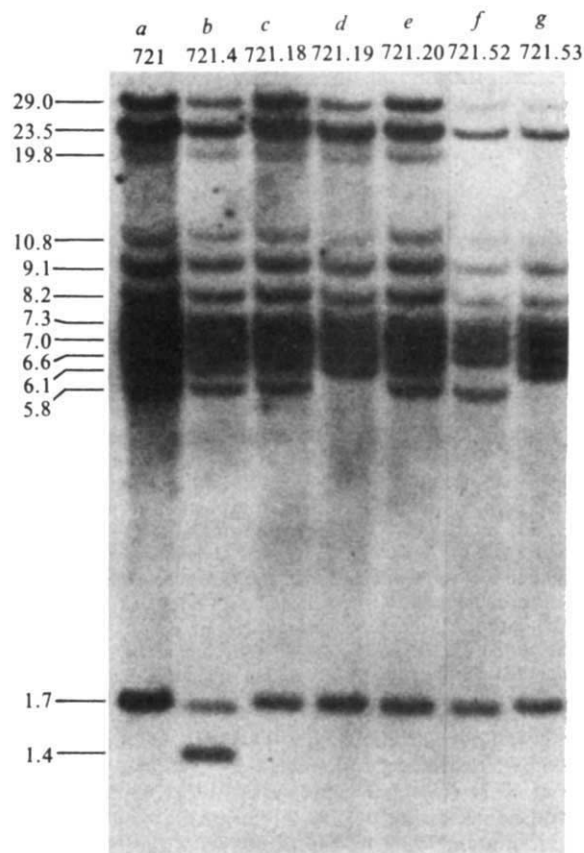


Fig. 2 Southern blot analysis of restriction endonuclease *Hind*III-digested genomic DNA from LCL 721 and some *HLA* loss mutants. 15 μ g of cellular DNA, isolated as described elsewhere¹⁸, plus 2 μ g of λ DNA as a control for digestion, were digested for 18 h with 30 units of *Hind*III, electrophoresed on a 0.7% agarose, and blotted by the Southern procedure¹⁹. The filter was baked for 2 h at 80 °C in a vacuum oven. Pre-hybridization²⁰ was in 10 ml of 50 mM NaPO₄ pH 6.5, 50% formamide, 5 $\times$ SSC, 5 $\times$ Denhardt's²¹, 1% glycine and 500 μ g ml⁻¹ salmon sperm DNA at 42 °C for 18 h. Hybridization²⁰ was in 10 ml of 20 mM Na phosphate pH 6.5, 50% formamide, 1 $\times$ SSC, 5 $\times$ Denhardt's, 100 μ g ml⁻¹ salmon sperm DNA, 10% dextran sulphate and 10⁷ c.p.m. of nick-translated¹⁷ pHLA-1 cDNA (specific activity 1.5 $\times$ 10⁸ c.p.m. ³²P per μ g) for 16 h at 42 °C. The filter was then washed in 1 $\times$ SSC for 1 h at 45 °C. Hybridizing bands were detected by autoradiography at -80 °C with a Dupont Lightning-Plus intensifying screen. Sizes of hybridizing bands are in kilobases based on the *Hind*III fragments of λ . Sources of DNA: Lane a, parental LCL 721; b, 721.4; c, 721.18; d, 721.19; e, 721.20; f, 721.52; g, 721.53. For *HLA* phenotypes see Table 1.

one chromosome 6 in mutant 721.19⁹. Mutant 721.53, which was derived from 721.19 by γ -ray irradiation and selection for cells that no longer expressed *HLA-B5*, still expresses *HLA-A2* and can be regarded as a *HLA-B* null mutant. Hybridization of pHLA-1 to *Hind*III-digested DNA from mutants 721.19 (Fig. 2d) and 721.53 (Fig. 2g) resulted in apparently identical banding patterns. Each mutant lost a 5.8-kb pHLA-1 hybridizing band that is present in LCL 721. Because both mutants have lost only the 5.8-kb band, its absence is correlated with the loss of *HLA-B5* expression in 721.53. The 5.8-kb band is present in the *HLA-B8* loss variants 721.4, -0.18 and -0.20. Therefore, the absence of the 5.8-kb band is more closely associated with the loss of *HLA-A1* expression than of *HLA-B8* expression. The other mutant which lost a pHLA-1 hybridizing band was the *HLA-A2*, *-B5*, *-DR1* loss mutant 721.52 (Fig. 2f). Compared with the 721 banding pattern, 721.52 has lost the 6.1-kb band. The loss of the 5.8-kb band in 721.19 compared with the loss of the 6.1-kb band in 721.52

Table 1 The HLA phenotype of LCL 721 and seven mutants isolated by immunoselection after irradiation of LCL 721 with γ -rays

Cell line	Phenotype					
	DR3	B8	A1	DR1	B5	A2
Parental 721	+	+	+	+	+	+
Variants						
4	+	-	+	+	+	+
18	+	-	+	+	+	+
20	+	-	+	+	+	+
19	-	-	-	+	+	+
52	+	+	+	-	-	-
53	-	-	-	+	-	+

The origin of LCL 721 and the procedures for inducing, isolating and characterizing mutants are described in ref. 9. A detailed description of the isolation of mutants 721.52 and 721.53 will appear elsewhere (R. DeM., in preparation). + Refers to expression of marker alleles. 721 and the mother of 721 were typed for HLA-C (C1-C6). Both expressed *HLA-C1* only. Mutant 721.52 did not express *HLA-C1*. This mutant lost expression of the *HLA-A2*, *HLA-B5* haplotype, inherited from the mother, and still expresses *HLA-A1* and *HLA-B8*. We conclude that 721 is heterozygous for *HLA-C*. *HLA-C1* is inherited from the mother on the *HLA-A2*, *HLA-B5* haplotype and something not definable from the father on the *HLA-A1*, *HLA-B8* haplotype.

shows that allelic variations within the *HLA* complex can be detected by a restriction endonuclease.

Loss of *HLA-B5* expression in mutant 721.53 did not result in an additional loss of pHLA-1 hybridizing bands in 721.53 relative to mutant 721.19. Moreover, two of the *HLA-B8* loss mutants, 721.18 (Fig. 2c) and 721.20 (Fig. 2e), had banding patterns identical to that of the parental line. The failure to detect alterations in these cases could result from DNA deletions that were too small to detect with our methods or that occurred in *HindIII* fragments that do not hybridize with pHLA-1. Alternatively, these lines may carry mutations that are not deletions, at least in the coding region of this gene(s). As pHLA-1 corresponds to only the COOH-terminal part of an *HLA* heavy chain and the 3'-noncoding region of the mRNA, substantial portions of an *HLA* class I gene could be missed when pHLA-1 is used as the probe. Probing with a nearly full-length *HLA* cDNA clone⁴ should reveal deletions in the remainder of the gene(s).

Detectable differences in intensity of some bands were also found. As evident from the ethidium bromide staining, some of these are likely to be due to slight differences in the amounts of DNA loaded. For example, more 721 DNA was loaded than from any of the mutants. However, equal amounts of DNA were loaded from mutants 721.18, 721.19 and 721.20 (Fig. 2, c-e). The decreased intensities of the 6.6-10.8-kb bands in 721.19 compared with those in 721.18 and 721.20 are, therefore, consistent with a greater loss of DNA in 721.19 than in 721.18 and 721.20; a complete haplotype in 721.19 compared with a single *HLA-B* allele in 721.18 and 721.20 (Table 1).

The results described here show the usefulness of using loss mutants in combination with a cDNA probe, pHLA-1, in analysing the organization of the human MHC. They support the concept that *HLA* class I genes are a multigene family. Similar results have been reported for the *H-2* class I gene family in inbred strains of mice^{6,8}. In this regard, note that mutant 721.19 had 11 of 12 pHLA-1 hybridizing bands found in 721. As this mutant has lost one complete *HLA* haplotype (from *HLA-A* to *HLA-DR*), it illustrates that heterozygosity at these loci in 721 has little effect on the number of *HindIII* bands detected with pHLA-1. The usefulness of radiation-induced *HLA* loss variants in the analysis of MHC structure is highlighted by the fact that of six variants analysed with one restriction enzyme and a single cDNA probe, three different alterations in the hybridization pattern were detected. Such alterations are not peculiar to *HindIII*. DNAs from mutants

721.19, 721.52 and 721.53 were also digested with *PvuII*, *PstI* and *SsrI* before probing with pHLA-1. The loss of a pHLA-1 hybridizing fragment was observed with each mutant and all the restriction enzymes (data not shown).

It was conceivable that the pHLA-1 hybridization pattern observed after digestion of DNA with *HindIII* resulted from reassociation of the 3'-untranslated portion of pHLA-1³ with a moderately repeated noncoding DNA sequence. Therefore, a *HindIII* digest of LCL 721 DNA was probed with a 600-bp fragment containing only the NH₂-terminal coding sequence of a nearly full-length *HLA* cDNA clone⁴. The results show that those pHLA-1-positive bands that are absent in mutants 721.19, 721.52 and 721.53 include the NH₂-terminal sequence (data not shown). Therefore, these bands react with probes for the COOH-terminus and the NH₂-terminus and may contain full-length *HLA* class I genes. The precise assignment of DNA fragments to specific *HLA* alleles will require more extensive analyses using newly available mutants, including some that have lost expressions of *HLA-A2* alone, *HLA-A2* and *-B5* or *HLA-A1* and *-B8*, as well as others that are *HLA-A*-null and *HLA-A*, *-B*-null (R. DeM. *et al.*, in preparation).

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A cloned T-cell line from a tolerant mouse represents a novel antigen-specific suppressor cell type

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Two major alternative models, clonal deletion and suppression, have been proposed as possible explanations for immunological tolerance^{1,2}. We have now isolated a functional T suppressor (T_s) cell clone to analyse the role of T_s cells in the induction and maintenance of tolerance. This cloned, long-term cultured T_s cell line (HF1) was derived from CBA/J mice (H-2^k) in which low-zone tolerance (LZT)³ had been induced by sub-immunogenic doses of bovine serum albumin (BSA). HF1 cells

show BSA-specific suppressor activity in two *in vitro* suppressor assays. HF1 T_h cells and a factor derived from them suppress unprimed, carrier-specific T helper (T_h) cells in an assay measuring IgG antibody production against fluorescein-conjugated BSA (Flu-BSA). Furthermore, they block DNA synthesis in an antigen-specific *in vitro* T-cell proliferation assay. Their proliferation in the presence of antigen-presenting cells is antigen specific and shows restriction to the I-A^k region of the major histocompatibility complex (MHC). In addition, HF1 T_h cells have a characteristic cytotoxic activity. The data reported suggest that clonal deletion and suppression models are not necessarily mutually exclusive. Clonal deletion could manifest itself by the action of T_h cells.

LZT to the antigens BSA and phage fd is accompanied and maintained by T_h cells⁴⁻⁶. Genetic control of LZT induction is, in at least one case, linked to the *Igh-1* allotype⁷. To analyse cellular interactions at the molecular level in tolerant animals, it is necessary to produce T-cell lines with specific immunological functions. In an attempt to produce an antigen-specific T_h cell line with the functional properties of T_h cells from low-zone-tolerant mice, CBA/J mice were tolerized by seven consecutive injections of 10 µg BSA per mouse. We anticipated that this sub-immunogenic dose of BSA would activate T_h cells but not T_h cells⁴, and such tolerogenic treatment should therefore pre-select T_h cells *in vivo* and endow them with the capacity to proliferate preferentially on subsequent *in vitro* selection. After

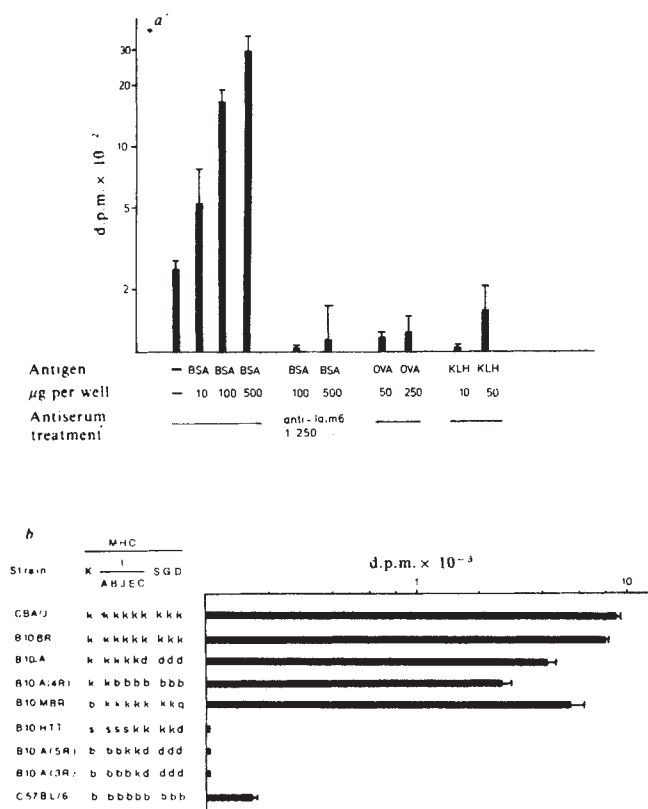


Fig. 1 Antigen specificity and I-A^k restriction in the proliferation of HF1 T_h cells. *a*, 10⁴ HF1 T_h cells were cultured in 250 µl of DME medium supplemented with 0.5% mouse serum in the presence of 5 × 10⁵ 3,000 R irradiated syngeneic spleen cells. Antigen-specific proliferation was measured by addition of BSA or ovalbumin (OVA) and keyhole limpet haemocyanin (KLH) as control antigens. In some experiments BSA-specific proliferation of HF1 T_h cells was inhibited by adding 1 µl monoclonal anti-Ia.m6 antibodies per well. ³H-TdR uptake was measured on day 4 of culture as described in Table 2 legend. *b*, 10⁴ HF1 T_h cells were cultured in 200 µl DME medium supplemented with 10% FCS and 10% CM in the presence of 5 × 10⁵ 3,000 R irradiated spleen cells from various congenic mouse strains, allowing mapping of I-A^k restriction. Proliferation of HF1 T_h cells was measured by ³H-TdR uptake on day 5 of culture as described in Table 2 legend.

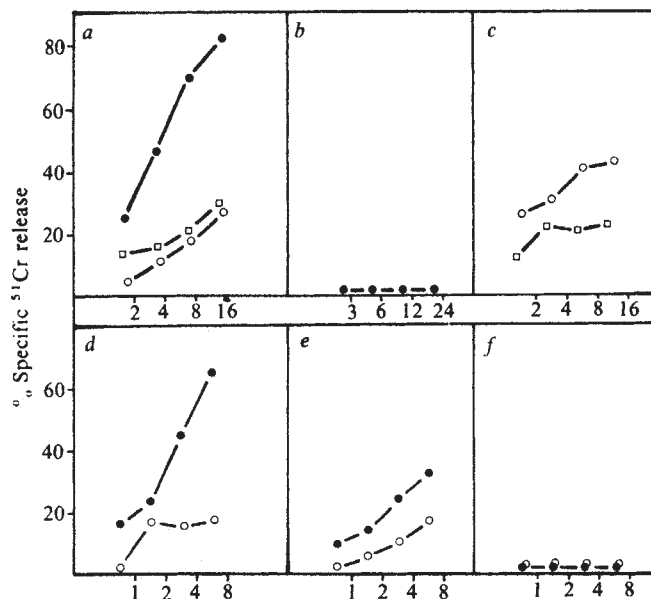


Fig. 2 Cytotoxicity of clone HF1 and subclone HF1.17 T_h cells against various target cells. Cytotoxicity was measured in a 6-h ⁵¹Cr-release assay using 2 × 10⁴ target cells per microtitre plate well. Per cent specific ⁵¹Cr release is plotted on the ordinate against the effector/target cell ratios on the abscissa. *a*, 2 × 10⁷ CBA spleen cells per ml were stimulated with 5 µg ml⁻¹ Con A in DME medium with 10% FCS for 2 days. Cells were washed once in balanced salt solution containing 10 µg ml⁻¹ α-methylmannoside and cultured 1 day longer in DME medium with 10% FCS in the presence of 5% CM. Lymphoblasts were purified on a Ficoll gradient, then labelled with ⁵¹Cr in the form of sodium chromate and used as target cells. HF1 T_h cells were tested in the absence (○) or presence (●) of 10 µg ml⁻¹ Con A. Target cell killing is observed in the absence of Con A but can be enhanced in the presence of Con A. HF1.17 cells were tested in the absence of Con A (□) or presence (●) of Con A. *b*, C57BL/6 EL4 T-lymphoma cells were used as targets. HF1 T_h cells cannot lyse these targets even in the presence (●) of Con A. This is an important finding because clone HF1 does not behave like T_c cells, which lyse target cells nonspecifically in the presence of Con A. *c*, 2 × 10⁷ CBA spleen cells per ml were cultured for 3 days in DME medium with 10% FCS in the presence of 40 µg ml⁻¹ LPS. Lymphoblasts were purified on a Ficoll gradient and used as target cells. Killing by HF1 (○) and HF1.17 (□) T_h cells is shown. Target cells used in the killing by HF1.17 T_h cells were further purified after Ficoll gradient separation by incubation on anti-Thy-1.2-coated Petri dishes. Non-adherent cells (>98% Ig⁺) were used in the ⁵¹Cr-release assay. *d*, BSA-specific long-term cultured T cells (LNC-BSA) were derived from lymph node cells of BSA-primed CBA/J mice. They were used as target cells and assayed for killing by HF1 T_h cells in the absence (○) or presence (●) of Con A. *e*, A BSA-specific T-cell clone (LNC-BSA.D.10) derived from LNC-BSA cells was used as target. Assay was in the absence (○) or presence (●) of Con A. *f*, A HGG-specific long-term cultured T-cell line (LNC-HGG) was used as target cells in the absence (○) or presence (●) of Con A. Lysis of LNC-BSA (*e*) but not of LNC-HGG (*f*) cells by clone HF1 cells suggests antigen specificity in killing.

the last *in vivo* antigenic treatment, splenic T cells were enriched by removing B cells on antibody-coated Petri dishes⁸. They were stimulated with antigen in bulk culture for 4 days and then cloned in soft agar⁹. HF1 cells were derived from 1 out of more than 100 colonies growing, and expanded. We have so far avoided virus transformation¹⁰ or T-cell hybridoma production¹¹ so as to study cellular functions both in terms of cellular interactions and factor production. The following surface characteristics were assessed in indirect immunofluorescence staining tests using fluorescein-conjugated rabbit anti-mouse or anti-rat immunoglobulin antibodies (Nordic): Thy-1.2⁺ (as defined by an AKR anti-CBA antiserum; Searle); Lyt-1⁺, 2⁺ (as defined by monoclonal antibodies, clones 53-7.3 and 53-6.7; Becton Dickinson); I-A^k and I-E^k (using monoclonal antibodies against Ia.m6, clone H 116-32.R5, and against Ia.m7, clone 13/4.R5; Camon). No I-J^k determinants could be detected by functionally active anti-I-J^k sera¹² from B10.A(3R) mice immunized with B10.A(5R) lymph node and spleen cells.

Table 1 Suppressor activity of long-term cultured, soft agar-cloned HF1 T_h cells and a factor derived from them in an *in vitro* suppressor assay

Cells in culture	Antigen in culture	Anti-Flu response (PFC per 10 ⁶ cultured cells)	
		IgM	IgG
8 × 10 ⁶ Flu-HGG primed SC + 1 × 10 ⁵ HF1	Flu-BSA	377 ± 14	79 ± 23
8 × 10 ⁶ Flu-HGG primed SC + 1 × 10 ⁵ 4CBA-C	Flu-BSA	271 ± 25	598 ± 27
8 × 10 ⁶ Flu-HGG primed SC + 1 × 10 ⁵ HF1	Flu-HGG	904 ± 16	1,339 ± 124
8 × 10 ⁶ Flu-HGG primed SC + 1 × 10 ⁵ 4CBA-C	Flu-HGG	810 ± 14	1,810 ± 80
8 × 10 ⁶ Flu-HGG primed SC + 1 × 10 ⁵ HF1.17	Flu-BSA	—	67 ± 21
8 × 10 ⁶ Flu-HGG primed SC + 1 × 10 ⁵ normal SC	Flu-BSA	—	301 ± 21
8 × 10 ⁶ Flu-HGG primed SC + 1 × 10 ⁵ HF1.17	Flu-RRBC	—	1,144 ± 64
8 × 10 ⁶ Flu-HGG primed SC + 1 × 10 ⁵ normal SC	Flu-RRBC	—	837 ± 44
7 × 10 ⁶ Flu-HGG primed SC + 1 × 10 ⁶ BSA-LZT SC	Flu-BSA	408 ± 10	<5
7 × 10 ⁶ Flu-HGG primed SC + 1 × 10 ⁶ normal SC	Flu-BSA	426 ± 6	439 ± 29
7 × 10 ⁶ Flu-HGG primed SC + 1 × 10 ⁶ BSA-LZT SC	Flu-HGG	349 ± 12	3,047 ± 63
7 × 10 ⁶ Flu-HGG primed SC + 1 × 10 ⁶ normal SC	Flu-HGG	641 ± 16	2,675 ± 79
8 × 10 ⁶ Normal SC	Flu-BSA	<5	<5
8 × 10 ⁶ Normal SC	Flu-HGG	16 ± 3	<5
8 × 10 ⁶ Flu-HGG primed SC + 1 × 10 ⁵ HF1	Flu-BSA	—	27 ± 20
8 × 10 ⁶ Flu-HGG primed SC + HF1 supernatant*	Flu-BSA	—	33 ± 9
8 × 10 ⁶ Flu-HGG primed SC	Flu-BSA	—	250 ± 29
8 × 10 ⁶ Flu-HGG primed SC + HF1.17 supernatant	Flu-BSA	—	69 ± 37
8 × 10 ⁶ Flu-HGG primed SC	Flu-BSA	—	182 ± 15

T_h line HF1 was produced in March 1980 in the following way. For induction of LZT to BSA, CBA/J mice received 2.5 mg hydrocortisone followed by seven consecutive injections of 10 µg BSA per mouse intraperitoneally (i.p.)⁵. One day after the last tolerogenic dose, splenic cells were incubated on Petri dishes coated with rabbit anti-mouse immunoglobulin antibodies⁸. The non-adherent T cells were removed and cultured in Costar plates (1.5 × 10⁶ cells per well) in the presence of syngeneic 3,000 R irradiated spleen cells (5 × 10⁵ cells per well) in a final volume of 1 ml Dulbecco's modified Eagle's (DME) medium supplemented with 5 × 10⁻⁵ M 2-mercaptoethanol, 2 mM glutamine, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin (DME medium) and 10% heat-inactivated horse serum (HS). At the beginning of culture, 10 µg BSA per ml were added to restimulate presumptive T_h cells *in vitro*. Four days later growing cells were cloned in soft agar⁹. On day 3 the agar was overlaid with 250 µl of a supernatant from Con A-stimulated rat spleen cells (conditioned medium, GM)²⁰. One to two weeks after starting the cloning procedure, colonies containing 40–100 cells were transferred to flat-bottomed microtitre wells and cultured in the presence of antigen (10 µg BSA per ml), 3 × 10⁴ syngeneic 3,000 R irradiated spleen cells and 10% CM in the same medium as described above. Cell cultures were fed every 3–4 days with antigen, 3,000 R irradiated syngeneic spleen cells and CM. After 4–6 weeks of continuous cell growth, proliferating cells were expanded on Costar plates or in Falcon 3013 tissue culture flasks. Instead of 10% HS and 10 µg ml⁻¹ BSA, 10% heat-inactivated fetal calf serum (FCS) (containing BSA as antigen) was now used. HF1 cells can be frozen in liquid N₂ and thawed with recovery of growth and function. Suppressor activity of HF1 cells was tested in an *in vitro* assay with Flu-BSA as antigen. This assay has been developed so as to have an IgG *in vitro* response which depends on primary T_h-cell activation: CBA/J mice were immunized i.p. with 2 mg Flu₄₀-HGG in complete Freund's adjuvant (CFA) on day 21 before culture and rechallenged with 2 mg Flu₄₀-HGG in Al(OH)₃ gel on day 7 before culture. Spleen cells from these animals served as a source of hapten-primed B cells. 8 × 10⁶ cells were co-cultured with 10⁵ HF1 cells. Cultures with 10⁵ cells of the alloreactive CBA/J anti-BALB/c (H-2^d) T-cell line 4CBA-C were used as control. Assays using spleen cells from CBA/J mice in which LZT to BSA had been induced served as further controls. Cells were cultured for 6 days in the DME culture medium with 10% HS and 7 µg Flu₄₀-BSA per ml, 10 µg Flu₄₀-HGG or 3 × 10⁶ fluoresceinated rabbit red blood cells (Flu-RRBC). In these culture conditions 10⁵ HF1 cells do not receive sufficient antigenic stimulus for proliferation. Direct and indirect plaque-forming cells (PFC) were assayed as described previously⁶. Mean ± s.d. of three PFC measurements is given. For the production of a functionally active suppressor factor from HF1 and HF1.17 cells, these were first adapted to proliferation in the absence of stimulator cells in DME with 10% FCS supplemented with 2.5% CM. Supernatants from 12-h cultures in serum-free conditions were used as the source of factor²¹. SC, spleen cells.

* Equivalent to 1 × 10⁵ cells.

Table 2 Suppressive activity of HF1 T_h cells in an antigen-specific *in vitro* T-cell proliferation assay

Lymph node cells in culture	Antigen or Con A in culture	Addition of	Incorporation of ³ H-thymidine (d.p.m.)
Expt 1			
BSA primed	—	—	8,953 ± 3,838
BSA primed	25 µg cBSA	—	51,120 ± 2,291
BSA primed	25 µg cBSA	3 × 10 ⁴ HF1 cells	12,963 ± 2,697
BSA primed	100 µg HGG	—	14,806 ± 6,268
HGG primed	—	—	4,578 ± 1,305
HGG primed	100 µg HGG	—	16,466 ± 917
HGG primed	100 µg HGG	3 × 10 ⁵ HF1 cells	15,892 ± 913
Expt 2			
BSA primed	—	—	9,615 ± 1,606
BSA primed	500 µg BSA	—	54,367 ± 4,120
BSA primed	500 µg BSA	2 × 10 ⁴ HF1 cells	17,818 ± 2,236
BSA primed	500 µg BSA	Anti-Ia.m6 1:250	14,541 ± 1,952
BSA primed	0.6 µg Con A	—	77,350 ± 2,244
BSA primed	0.6 µg Con A	Anti-Ia.m6 1:250	99,044 ± 2,281

The T-cell proliferation assay using inguinal lymph node cells from antigen-primed CBA/J mice was used as described elsewhere¹³. 2 × 10⁵ lymph node cells were cultured in flat-bottomed microtitre wells with 3 × 10⁵ 3,000 R irradiated syngeneic spleen cells in 250 µl of DME medium containing 0.5% mouse serum. At the beginning of culture they were stimulated with either 25 µg heat-aggregated BSA (cBSA) or 100 µg HGG (expt 1) and 500 µg BSA or 0.6 µg Con A (expt 2) per well. Antigen-specific suppressor activity of HF1 cells was determined by addition of 2 × 10⁴ or 3 × 10⁴ cells per well. The same degree of suppression was also demonstrated if HF1 cells were treated with mitomycin C before addition to culture (data not shown). Monoclonal anti-Ia.m6 antibodies (1 µl per well) were added to demonstrate its influence on BSA-specific T-cell proliferation. Proliferation was measured on day 4 by pulsing the culture with 0.4 µCi ³H-thymidine (³H-TdR) for 16 h and measuring ³H-TdR uptake. For each experimental condition three independent cultures were set up and mean d.p.m. ± s.d. are given. Lymph node cells were taken from CBA/J mice immunized with 1 mg BSA or 1 mg HGG per mouse in CFA subcutaneously at the base of the tail. Animals were not used until 10 days after immunization.

HF1 cells are Ig⁻ (direct immunofluorescence staining with fluorescein-labelled rabbit anti-mouse Ig antibodies) and FeR⁻ (binding of aggregated fluoresceinated human globulin, Flu-HGG).

HF1 T_h cells are functionally active, as demonstrated by suppression of IgG but not IgM anti-Flu antibody production after stimulation with Flu-BSA in an *in vitro* suppressor assay using unprimed T_h cells and Flu-primed B cells. This suppression is comparable with the effect of BSA-specific T_h cells from LZT mice (Table 1). In an independent assay, HF1 cells suppressed antigen-specific BSA-induced *in vitro* proliferation of lymph node cells¹³ from BSA-primed CBA/J mice. This antigen-specific proliferation was also inhibited by monoclonal anti-I-A^k antibodies in place of HF1 T_h cells, indicating that stimulation is I-A^k restricted (Table 2). This BSA-induced *in vitro* T-cell proliferation assay was used to analyse subclones of HF1 T_h cells. HF1 T_h cells were subcloned by limiting dilution microculture (1 and 0.3 cells per microplate well) in the same culture conditions as described in Fig. 1b legend. Plating efficiencies ranged from 70 to 100%. All 22 subclones expanded and tested for functional activity showed suppressive activity (data not shown), indicating that HF1 T_h cells represent a homogeneous population.

Restriction analysis was performed to identify genes of the H-2 region which regulated proliferation of HF1 T_h cells in the presence of antigen. We first established that the proliferation of HF1 T_h cells requires antigen in the presence of irradiated syngeneic stimulator cells (Fig. 1). This proliferation can be inhibited by supplementing the cultures with monoclonal anti-I-A^k antibodies. Mapping experiments show that their proliferative response is restricted to I-A^k recognition in the MHC. An influence of the I-J^k region is excluded (Fig. 1).

I-A^k determinants on HF1 T_s cells can be labelled biosynthetically using ³⁵S-methionine (unpublished data), showing that they are synthesized by HF1 T_s cells themselves. These data might be a key to understanding differences in the antigen-dependent activation processes of T_h and T_s cells. Expression of I-A determinants on cloned T_h cells has not been found, but available evidence from heterogeneous T_h-cell populations suggests that there is little expression of I-A determinants on these cells.¹⁴ One could envisage a competitive intervention of T_s cells in the interaction between antigen-presenting, I-A determinant-bearing macrophages and T_h cells. The suppressor effect of HF1 T_s cells can also be demonstrated using cell-free supernatants from HF1 T_s cells. The factor both suppresses IgG anti-Flu antibody production *in vitro* on challenge with Flu-BSA (Table 1) and inhibits BSA-specific T-cell proliferation (data not shown). The factor has not been analysed in detail and it remains to be seen whether it is different from the suppressor factor produced by a T_s-cell clone from hyperimmune mice.¹⁵

The additional cytotoxic activity of HF1 T_s cells (Fig. 2) distinguishes them from all other T_s-cell lines described so far. HF1 T_s cells can kill concanavalin A (Con A)- or lipopolysaccharide (LPS)-stimulated syngeneic spleen cells but not allogeneic target cells, even in the presence of Con A. Cytotoxic T (T_c) cells nonspecifically lyse target cells in the presence of Con A, due to cell-cell bridging^{16,17}. This implies that the mechanism of cytotoxicity through HF1 T_s cells is different from T_c cell activity.

Subclone HF1.17 isolated from microcultures seeded with an average of 0.3 HF1 cells per well has cytotoxic activity (Fig. 2) and produces a suppressive factor (Table 1). As we were unable to demonstrate cytolysis by the factor itself, a cloned cell of the HF1 T_s line must possess two functional properties, factor-mediated suppression and cell-mediated cytotoxicity.

In considering implications of the above findings for possible mechanisms of immunological tolerance, one must be aware that no definite proof has been found for the absence of T_s cells in cases of apparent clonal deletion¹⁸. A presumed incompatibility of the extremes of suppression and deletion models is conceptually largely based on the assumption that clonal deletion is due to a direct interaction of antigen with the cell to be rendered tolerant¹⁹. Our finding of a novel type of T_s cell clone provides an experimental basis for a combination of both models, suggesting that clonal deletion could be a consequence of T_s activity.

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Mouse spleen and IgD-secreting plasmacytomas contain multiple IgD δ chain RNAs

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Immunoglobulin gene expression requires that cells combine the correct genetic information by DNA rearrangement and RNA processing to produce mature, translatable mRNAs. The mechanisms that operate to produce IgD heavy chain (δ chain) mRNA are thought to be especially complex¹⁻³. Like other immunoglobulins, IgD exists in membrane and secreted forms^{4,5}. By analogy with results of studies of μ ⁶⁻¹⁰ and γ ^{11,12} gene expression, we expected that δ chains would also be encoded by two different mRNAs, containing common sequence information transcribed from constant region gene segments but different information transcribed from coding sequences located 3' to the constant region gene segments. Instead, we identified¹³ more than two δ mRNAs in normal mouse spleen and in two IgD-secreting plasmacytomas¹⁴, TEPC 1017 and TEPC 1033. Now we have used ³²P-labelled DNA fragments prepared from a δ cDNA clone¹⁵ and a δ genomic clone¹⁶ to characterize by hybridization these δ RNAs, fractionated on methyl mercury hydroxide agarose gels. We find that normal mouse spleen contains a major 2.9-kilobase (kb) and a minor 2.1-kb RNA encoding membrane-bound δ chains (δ_m) and that TEPC 1017 and TEPC 1033 contain similar δ_m RNAs plus a 1.75-kb RNA encoding secreted δ chains (δ_s). We have also observed less abundant δ RNA species (2.65 and 3.2 kb). Different gene segments or combinations of segments are used in the 3' termini of the multiple δ RNAs.

The δ chain synthesized by normal spleen cells bearing IgD on their surface is presumed to be derived from a complex transcription unit, including a rearranged μ gene^{1-3,17}. In contrast, synthesis of δ chain for secreted IgD is postulated to require deletion of the μ gene^{1-3,17}. Figure 1 shows that TEPC 1017 contains most of its constant domains on a 3.3-kilobase pair (kbp) *Hind*III fragment, which is smaller than that of liver (4.0 kbp), and that TEPC 1033 contains its δ on a larger (4.6 kbp) fragment. These data are compatible with the relocation of different V-D-J complexes 5' to the C δ 1 domain, between the *Hind*III site 2.0 kbp 5' to C δ 1 and the *Eco*RI site 0.5 kbp 5' to C δ 1 in these plasmacytomas¹⁶.

DNA rearrangements 3' to expressed constant region genes have also been reported in plasmacytomas¹⁸, though only rarely. Figure 1 demonstrates that C δ genes in liver and both plasmacytomas are contained in a single 9.8-kbp *Eco*RI fragment. This fragment, which was isolated from BALB/c liver and cloned, has previously been shown to contain the C δ gene segments¹⁶ plus virtually all the 3' gene segments to be discussed here¹⁷. Thus, no detectable rearrangements 3' to the δ constant domains have occurred in these tumours, although the possibility of small deletions or rearrangements within the 9.8-kbp fragment cannot be excluded. We assume that (1) this portion of the DNA is identical in liver, the IgD-secreting plasmacytomas and IgD-bearing cells in normal spleen; and (2)

all the δ RNAs we observe in the plasmacytomas and in normal spleen are derived from δ gene transcription units identical in their 3' sequence to that found in liver DNA.

The 1.75-kb δ RNA identified in TEPC 1017 and TEPC 1033 RNA is the most abundant δ RNA in these plasmacytomas and is not detected in normal spleen RNA (Fig. 2a). Both plasmacytomas secrete large amounts of IgD protein¹⁴; spleen cells bear IgD on their surfaces but fewer than 1 in 10⁵ cells secrete IgD¹⁹. Thus, it is likely that the 1.75-kb RNA species is mRNA encoding δ chain for secreted IgD and that one or more of the less abundant δ RNA species in plasmacytomas are mRNAs encoding δ chain for membrane-bound IgD.

The hybridization data presented in Fig. 2 and summarized in Fig. 3 show that the only gene segment to the 3' side of C δ 3 represented in the 1.75-kb δ RNA is contained in the DC1 fragment. DNA sequence data¹ reveal that the exon contained in the DC1 fragment encodes a non-hydrophobic polypeptide consistent with the carboxy-terminal sequence of a secreted form of heavy chain.

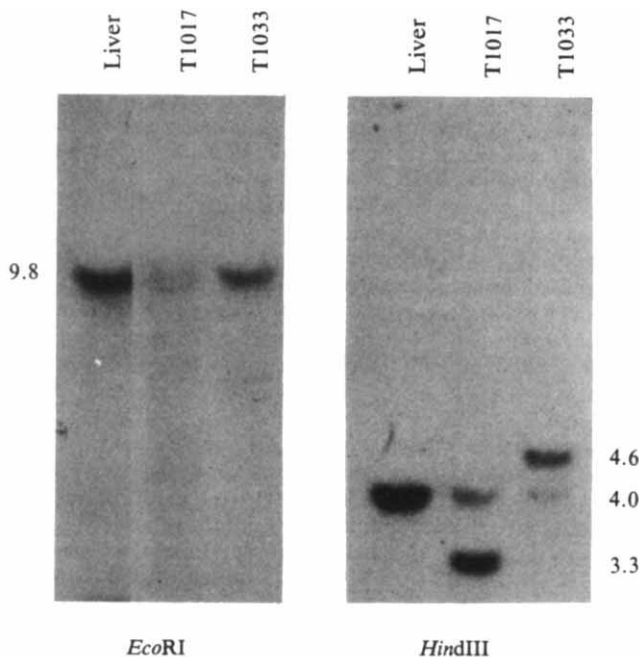


Fig. 1 C δ hybridization analysis of *Eco*RI- and *Hind*III-digested genomic DNA from BALB/c mouse liver and TEPC 1017 and TEPC 1033 tumours. Fragments generated by digestion of 25 μ g of each DNA were fractionated by electrophoresis in 0.7% agarose gels, denatured in alkali, neutralized and transferred to a sheet of nitrocellulose²⁴. The sheet was hybridized to a 563-bp DNA fragment (containing only C δ sequences) that had been excised from p δ 54J plasmid¹⁵ by digestion with *Pvu*II and *Hind*III, purified by electrophoresis on a 4% polyacrylamide gel²⁵ and labelled with ³²P by nick-translation²⁶ to 1.0 $\times$ 10⁸ c.p.m. μ g⁻¹ of which 2.5 $\times$ 10⁶ c.p.m. were used in 25 ml hybridization solution. Lengths of hybridizing fragments (in kbp) were determined by comparison with ethidium bromide-stained *Eco*RI and *Hind*III fragments of phage λ DNA and are indicated. The pattern of δ -hybridizing fragments in liver DNA is consistent with the previously published restriction map of the δ gene cloned from BALB/c liver¹⁶; immunoglobulin genes in liver DNA are assumed to remain in germ-line configuration. Both tumours have ~25% of their δ DNA in the form seen in liver as determined by densitometric scanning of the autoradiogram. Both plasmacytomas are pseudotetraploid (G. Klein and S. Ohno, personal communication), and it seems likely that one of the four chromosomes remains unrearranged while the other three have undergone rearrangement. However, because the plasmacytoma DNAs were prepared from solid tumours, it is possible that contaminating normal tissue contributed some or all of the unrearranged genes seen in these digests.

The only other δ RNA species that hybridizes with DC1 is the much less abundant 3.2-kb δ RNA in TEPC 1017 and TEPC 1033 (Fig. 2c). This RNA also hybridizes with the AC1 probe (Fig. 2b), and it may represent a short-lived precursor of the 1.75-kb δ_s mRNA.

Membrane IgD is present on most splenic B cells²⁰ and on TEPC 1017 and TEPC 1033 (ref. 14). We presumed that δ_m mRNA would be the most abundant δ RNA in spleen as well as being readily detectable in RNA from these plasmacytomas. The 2.9-kb δ RNA meets these criteria (Fig. 2a) and probably encodes δ chain for membrane IgD. This 2.9-kb δ_m mRNA hybridizes with VDC1 (Fig. 2e) and VDC2 (Fig. 2f) but not with DC1 (Fig. 2c). The DNA sequence¹⁷ of VDC1 encodes a hydrophobic polypeptide ~50% homologous to the amino acid sequence of the δ_m transmembrane polypeptide. This is further evidence that the 2.9-kb δ RNA is a form of δ_m mRNA.

A less abundant δ RNA (2.1-kb) in the plasmacytomas and in spleen also hybridizes with VDC1 sequences. It probably represents another form of δ_m mRNA lacking sequences encoded in VDC2 (Fig. 2f) or VDC3 (data not shown) which are found in the 2.9-kb δ_m mRNA.

Whereas the 2.9-kb δ RNA in the plasmacytomas hybridizes with AC1 (Fig. 2b), the 2.9-kb δ RNA from spleen does not. Thus, plasmacytoma cells may contain at least two δ RNAs in the 2.9-kb band: one (containing AC1 sequences) may be a precursor of the 2.1-kb δ_m mRNA, and another (similar to that in spleen) may be an alternative form of δ_m RNA containing a different 3'-untranslated region.

The 3.2-kb δ RNA species is rare in both spleen and plasmacytomas. As discussed above, in the plasmacytomas it appears to be a form of δ_s RNA. The 3.2-kb δ RNA in the spleen hybridizes with VDC1, VDC2 and VDC3 (Fig. 2e, f) but not with DC1 (Fig. 2c), suggesting that it is a form of δ_m RNA, either a third mature form of δ_m mRNA in the spleen, or a precursor of one of the other two δ_m mRNAs.

The DC2 probe hybridizes (Fig. 2d) with a 2.65-kb RNA species but with none of the other δ RNAs described here. The amount of the 2.65-kb RNA species varies among different preparations of plasmacytoma and spleen RNA and is by far the least abundant δ RNA detected. Thus the composition of this δ RNA with respect to other DC, AC or VDC sequences and its possible function cannot yet be stated.

Our studies of normal spleen and two IgD-secreting plasmacytomas suggest that δ chain is unusual among immunoglobulins in that its mRNAs exist in so many forms. While some of these δ RNAs may represent aberrant transcripts analogous to the 'C μ RNAs' described by others^{21,22}, their presence in normal spleen argues against their being abnormal. Preliminary experiments have identified the 2.1-, 2.9- and 3.2-kb δ RNAs on polyribosomes prepared from B-cell lymphomas which also indicates that all three are normal, functional mRNAs. The complexity of the δ gene transcripts contrasts with the mouse IgM^{8,9} and IgG^{11,12} heavy chains, each of which is encoded by only two mRNAs, one for the membrane form and one for the secreted form of the protein.

Other groups^{2,3} have studied δ RNA in B-cell tumours and hybridomas, but none has reported the complex array of δ RNAs identified here in normal spleen and plasmacytomas. The 1.8-kb δ RNA reported by these groups is probably identical to the δ_s mRNA we report. Similarly, their 2.7-kb mRNAs^{2,3} may be identical to the 2.9-kb δ_m mRNAs we report here. The less abundant RNAs reported here of 2.65 kb and 3.2 kb have not been described by other groups, although Maki *et al.*³ noted that R-loops observed but not presented suggested that there could be other forms of δ RNA.

We have identified δ RNAs in spleen and TEPC 1017 and TEPC 1033 which encode the membrane form(s) of δ chain and share certain significant characteristics. Most important, a hydrophobic (transmembrane) polypeptide¹⁷ is encoded in both the 2.9- and the 2.1-kb δ_m mRNAs found in spleen and the plasmacytomas. In addition, the 2.9-kb δ_m mRNAs from both sources contain sequences encoded in VDC2 and VDC3.

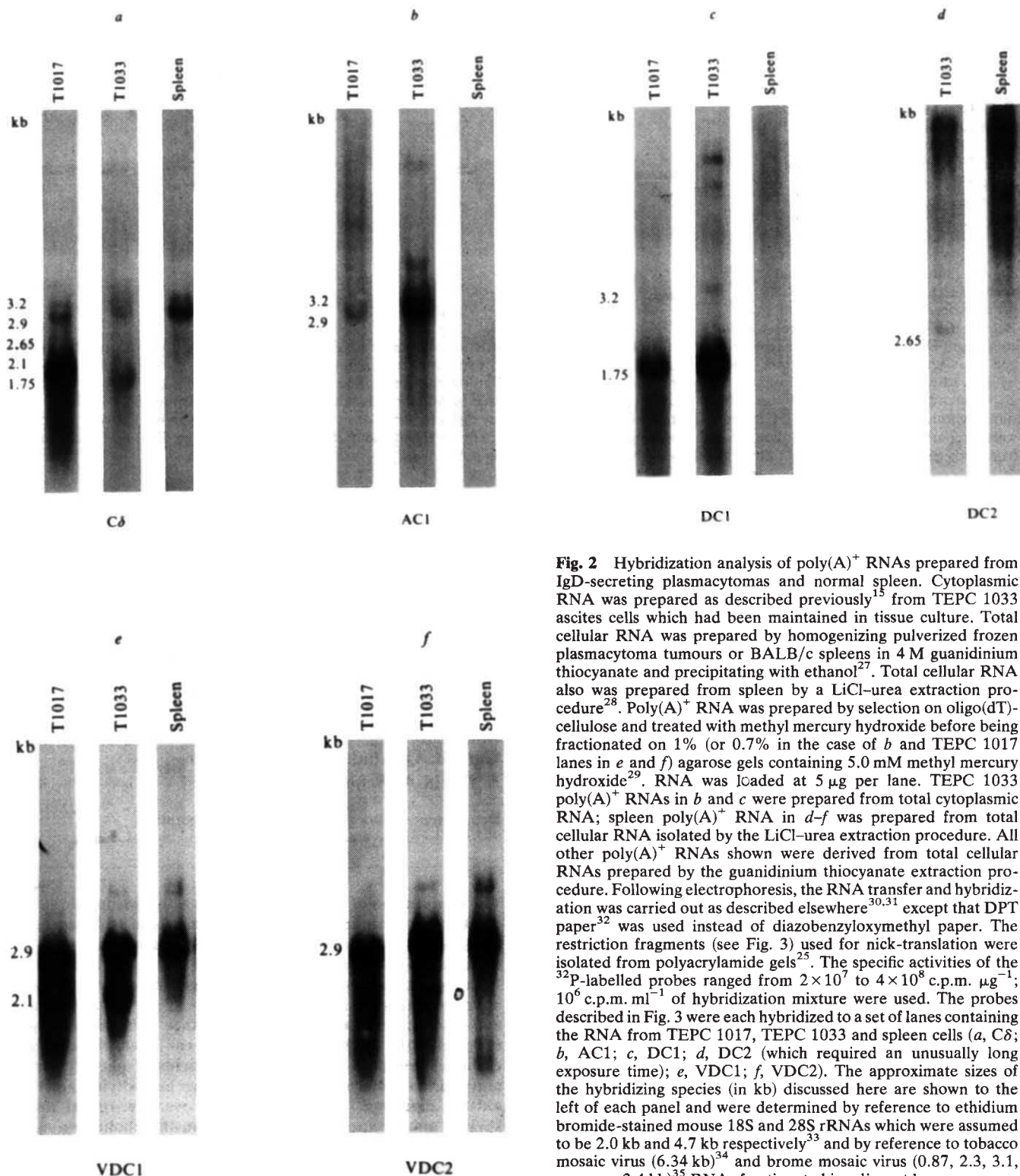


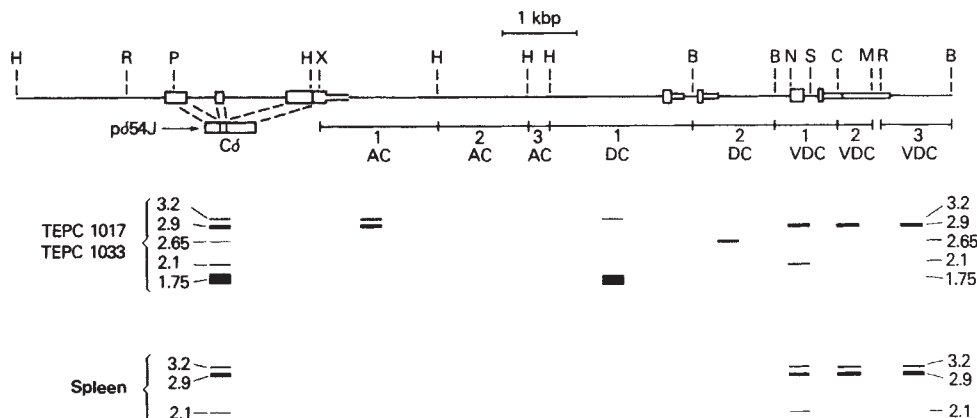
Fig. 2 Hybridization analysis of poly(A)⁺ RNAs prepared from IgD-secreting plasmacytomas and normal spleen. Cytoplasmic RNA was prepared as described previously¹⁵ from TEPC 1033 ascites cells which had been maintained in tissue culture. Total cellular RNA was prepared by homogenizing pulverized frozen plasmacytoma tumours or BALB/c spleens in 4 M guanidinium thiocyanate and precipitating with ethanol²⁷. Total cellular RNA also was prepared from spleen by a LiCl-urea extraction procedure²⁸. Poly(A)⁺ RNA was prepared by selection on oligo(dT)-cellulose and treated with methyl mercury hydroxide before being fractionated on 1% (or 0.7% in the case of *b* and TEPC 1017 lanes in *e* and *f*) agarose gels containing 5.0 mM methyl mercury hydroxide²⁹. RNA was loaded at 5 µg per lane. TEPC 1033 poly(A)⁺ RNAs in *b* and *c* were prepared from total cytoplasmic RNA; spleen poly(A)⁺ RNA in *d-f* was prepared from total cellular RNA isolated by the LiCl-urea extraction procedure. All other poly(A)⁺ RNAs shown were derived from total cellular RNAs prepared by the guanidinium thiocyanate extraction procedure. Following electrophoresis, the RNA transfer and hybridization was carried out as described elsewhere^{30,31} except that DPT paper³² was used instead of diazobenzoyloxymethyl paper. The restriction fragments (see Fig. 3) used for nick-translation were isolated from polyacrylamide gels²⁵. The specific activities of the ³²P-labelled probes ranged from 2 × 10⁷ to 4 × 10⁸ c.p.m. µg⁻¹; 10⁶ c.p.m. ml⁻¹ of hybridization mixture were used. The probes described in Fig. 3 were each hybridized to a set of lanes containing the RNA from TEPC 1017, TEPC 1033 and spleen cells (*a*, Cδ; *b*, AC1; *c*, DC1; *d*, DC2 (which required an unusually long exposure time); *e*, VDC1; *f*, VDC2). The approximate sizes of the hybridizing species (in kb) discussed here are shown to the left of each panel and were determined by reference to ethidium bromide-stained mouse 18S and 28S rRNAs which were assumed to be 2.0 kb and 4.7 kb respectively³³ and by reference to tobacco mosaic virus (6.34 kb)³⁴ and brome mosaic virus (0.87, 2.3, 3.1, 3.4 kb)³⁵ RNAs fractionated in adjacent lanes.

Different portions of these 'very distant' 3' sequences may be used in the 3'-untranslated regions of these δ_m mRNAs analogous to the variable 3'-untranslated regions of the multiple forms of mRNA for mouse dihydrofolate reductase²³. Alternatively, one or more exons in the sequence may be translated and expressed as alternative or additional 3' polypeptide termini. Such different forms of δ_m chain might thus allow not only a transmembrane 'anchor' for membrane IgD but also one or more internal (cytoplasmic) termini available for different effector functions of IgD. Another possibility is that the multiplicity of δ_m mRNAs represents a redundancy in the δ gene to

ensure that B cells will be able to produce membrane IgD even if genetic accidents interfere with mRNA processing and/or translational pathways.

Note, however, that at least some of the δ_m mRNAs in the plasmacytomas are different from those identified in normal spleen. AC1 and DC1 sequences are readily detectable in the plasmacytoma RNAs but not in normal spleen. The lack of AC1 and DC1 hybridization in spleen RNA probably reflects the fact that spleen primarily synthesizes δ_m and contains very few plasma cells which utilize RNA transcriptional pathways leading to δ_s expression. It has been postulated^{2,3} that deletion

Fig. 3 Restriction enzyme map showing the BALB/c δ gene¹⁶ and the genomic fragments subcloned from it. B, *Bam*HI; C, *Cla*I; H, *Hind*III; M, *Mbo*I (converted to *Bam*HI when inserted into Charon 28 phage clone); N, *Hinc*II; P, *Pvu*II; R, *Eco*RI; S, *Rsa*I; and X, *Xba*I. The restriction enzyme map and the boxes defining δ exons are described in greater detail elsewhere^{16,17}. Fragments from phage clones of liver DNA¹⁶ indicated diagrammatically here were subcloned into appropriate plasmid vectors. The preparation of the C δ probe is described in Fig. 1 legend. Before nick-translation²⁶ the recombinant DNA fragments were separated from the plasmid vector by restriction endonuclease digestion and fractionation on polyacrylamide gels²⁵. A semiquantitative diagram summarizing the results of repeated experiments in which plasmacytoma RNA or spleen RNA was hybridized with each probe is presented below the name of each probe. Photographs of representative autoradiograms are presented in Fig. 2, but many of the minor bands are difficult to photograph in the presence of other bands which hybridize very strongly. VDC3 gave the same hybridization pattern as VDC2, and AC2 and AC3 did not reveal any discrete bands of hybridization (data not shown). Two fragments were prepared from VDC1 that contain the following sequences: those between *Hinc*II and *Rsa*I, or those between *Rsa*I and *Cla*I. Both fragments gave the same pattern of hybridization as the whole VDC1 (data not shown).



of the μ gene is necessary for cells to synthesize secreted IgD. The rearrangement 5' to C δ 1 in TEPC 1017 and TEPC 1033 is consistent with such a deletion, and it could also alter transcriptional and processing pathways in the δ_m gene complex.

Membrane IgD has long been thought to be important in B-cell development although its exact role remains controversial. As discussed above, the multiple δ_m mRNAs described in this report could represent mRNAs for multiple forms of δ_m protein. Cloning of DNA sequences for these multiple δ_m mRNAs and cell-free translation studies are in progress to help elucidate the biological role of membrane IgD and the regulation of its expression.

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Xenobiotic conjugation: a novel role for bile acids

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The conjugation of foreign compounds with hydrophilic natural products (for example, amino acids, glucuronic acid) represents an important biochemical mechanism of attenuating xenobiotic toxicity and of facilitating excretion of hydrophobic chemicals. Although several conjugation reactions are known¹, bile acids have not been implicated previously in the conjugation of xenobiotics. The physiological importance of natural bile acids is based partly on their propensity to conjugate through their carboxyl groups with several amino acids (especially glycine and taurine), thereby creating effective emulsifying agents to aid the digestion of lipids. However, we now report that in several animal species, bile acids form metabolic conjugates through a hydroxyl group with an acidic metabolite of fluvalinate, a pyrethroid insecticide. Conjugates of this acidic metabolite with cholic, taurochenodeoxycholic and taurocholic acids are major secondary metabolites found in excreta of the cow, chicken and rat, respectively, and represent 5–12% of the faecal ¹⁴C-labelled residue.

Fluvalinate (α -cyano-3-phenoxybenzyl 2-(2-chloro-4-trifluoromethyl-anilino)-3-methylbutanoate; Mavrik) is a pyrethroid insecticide² that is metabolized primarily by ester hydrolysis to an anilino acid (Table 1 and Fig. 1), and is excreted intact, conjugated with amino acids, or hydroxylated at the isopropyl methyl group^{3,4}. Using TLC and reverse-phase liquid chromatography, we isolated from cow faeces an unknown metabolite of fluvalinate which was identified by mass spectrometry as an anilino acid conjugate of cholic acid (Table 1).

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Table 1 Bile acid conjugates of an acidic xenobiotic in animal faeces

Conjugate	R ₁	R ₂	R ₃	Rat (0–3 day)	% ¹⁴ C in faeces Chicken (0–1 day)	Cow (3–4 day)
Cholate	OH	OH	O [−]	0.5	<0.5	8.1*
Chenodeoxycholate	OH	H	O [−]	—	—	1.3
Deoxycholate	H	OH	O [−]	—	—	2.2
Taurocholate	OH	OH	NHCH ₂ CH ₂ SO ₃ [−]	5.0	<0.5	<0.5
Taurochenodeoxycholate	OH	H	NHCH ₂ CH ₂ SO ₃ [−]	3.2	11.9	<0.5
Taurodeoxycholate	H	OH	NHCH ₂ CH ₂ SO ₃ [−]	<0.5	<0.5	<0.5
Glycocholate	OH	OH	NHCH ₂ CO ₂ [−]	<0.5	<0.5	0.3
Glycochenodeoxycholate	OH	H	NHCH ₂ CO ₂ [−]	<0.5	<0.5	0.6
Glycodeoxycholate	H	OH	NHCH ₂ CO ₂ [−]	<0.5	<0.5	0.2

* The cholic acid conjugate when methylated (diazomethane in ether/methanol) displayed the following characteristics: TLC (silica gel GF), $R_F = 0.28$ in ether/hexane (1:1); reverse-phase liquid chromatography (LiChrosorb RP-8 column, acetonitrile/0.1% acetic acid (75:25), flow rate 1.6 ml min^{−1}, UV detection at 254 nm, $k' = 12.7$); mass spectrum (m/z (relative intensity), electron impact), 701 (1.1, M⁺ for Cl = 37), 699 (3, M⁺ for Cl = 35), 252 (97), 250 (100); mass spectrum (chemical ionization (CH₄)), 728 (5, M + 29), 700 (6, M + H for Cl = 35).

† The taurochenodeoxycholic acid conjugate (as its methyl ester) was purified by TLC (silica gel GF, ethyl acetate/hexane (2:1)) and reverse-phase liquid chromatography (methanol/0.1% acetic acid (80:20), $k' = 15.5$). It was identified from the following spectral data: ¹H NMR (Bruker WM-300, 300 MHz, (CDCl₃)), $\delta = 0.66$ (s (singlet), 3, CH₃ at C-18); 0.91 (s, 3, CH₃ at C-19); 1.03 (d (doublet), 3, $J = 7$ Hz, isopropyl CH₃); 1.08 (d, 3, $J = 7$ Hz, isopropyl CH₃); 3.33 (t (triplet), 2, $J = 6$ Hz, CONHCH₂); 3.74 (t, 2, $J = 6$ Hz, CH₂SO₃); 3.93 (s, 3, OCH₃); 4.66 (multiplet, 1, H at C-3 of steroid). Field desorption mass spectrum (m/z (relative intensity)), 792 (59, M⁺ for Cl = 37), 790 (53, M⁺ for Cl = 35), 774 (81, M⁺ − H₂O for Cl = 37), 772 (100, M⁺ − H₂O for Cl = 35). Several reference compounds were synthesized for analysis by ¹H NMR spectroscopy (Varian T-60 instrument, CDCl₃) to determine the position of conjugation on the bile acid. For each standard the chemical shift of the methine hydrogen in the bile acid at the carbon bearing an acylated hydroxyl was used to diagnose the site of esterification: for the anilino acid conjugate of methyl lithocholate (3 β -methine hydrogen only), $\delta = 4.80$ p.p.m. (broad singlet); for the trisbenzoate of methyl cholate, $\delta = 4.82$, 5.38, 5.50 (3 β -, 7 β - and 12 β -methine hydrogens, respectively); for the bisbenzoate of methyl deoxycholate, $\delta = 4.90$ and 5.43 (3 β - and 12 β -methine hydrogens, respectively). Together with the broadness of the 3 β -methine hydrogen resonance (compared with that of the 7 β - and 12 β -hydrogens), these data support our assignment of conjugation at the 3 α -hydroxyl moiety.

Subsequent comparison with a synthetic standard confirmed this.

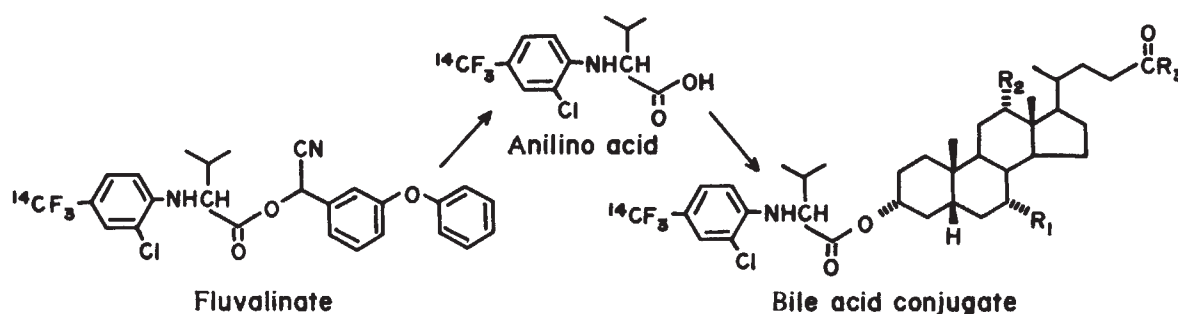
The apparent novelty of this bile acid adduct led us to examine the generality of the finding. Most (67%) of the ¹⁴C-labelled metabolite residue in excreta of chickens treated with [CF₃-¹⁴C]fluvialinate was highly polar (no migration on silica gel TLC with development in ethyl acetate). However, methylation of this polar ¹⁴C-residue with diazomethane gave (in part) a relatively nonpolar product ($R_F = 0.25$ on silica gel GF TLC developed with ethyl acetate/hexane, 2:1). By administering a single oral dose of ¹⁴C-fluvialinate (100 mg per kg) to a Leghorn hen, we purified 300 μ g of this metabolite (as its methyl ester) using TLC and reverse-phase liquid chromatography. The metabolite was identified as an anilino acid conjugate of taurochenodeoxycholic acid by ¹H NMR spectroscopy and field desorption mass spectrometry. Acylation of taurochenodeoxycholic acid by the anilino acid occurred at the 3 α -hydroxyl group (not the 7 α -hydroxyl), as deduced from the ¹H NMR chemical shift of the methine hydrogen at C-3 of the acylated bile acid ($\delta = 4.66$ p.p.m.) and its multiplet coupling pattern. A synthetic sample of the conjugate gave an identical NMR spectrum, showing that chemical and metabolic acylation both favour the least hindered 3 α -hydroxyl position of the bile acid⁵.

To determine any additional bile acid conjugates, the remaining common bile acids (as methyl esters) were acylated using

the acyl chloride of the anilino acid, thus providing reference standards for chromatographic comparison. Rats, chickens and a cow were given a single oral dose of fluvialinate at 1 mg per kg; analysis of their excreta by TLC, and reverse-phase and normal-phase liquid chromatography, revealed a general occurrence of these bile acid conjugates (Table 1). The relative abundance of a particular conjugate appeared to be proportional to the composition of the natural bile acids in the animal: for example, taurochenodeoxycholic acid is the major bile acid in chickens while taurocholic and taurochenodeoxycholic acids are most abundant in rats⁶.

The fact that bile acid conjugates of xenobiotics have not been described previously may be explained by the numerous difficulties in manipulating these conjugates. In this study the taurine-containing conjugates were most abundant—these compounds are highly polar because of the free sulphonic acid moiety, adhering tightly to silica gel and even laboratory glassware. Methylation of the sulphonic acid is inefficient and once formed, the methyl ester decomposes slowly whether neat or in acetone solution.

We have described a new, apparently general role for bile acids in the metabolic conjugation of a xenobiotic by a rodent, a ruminant and a bird. Although this example involves a pesticide metabolite, certain drugs (or their acidic metabolites) may be processed similarly. Indeed, normal resorption of these bile acid conjugates may offer an alternative

**Fig. 1** Conjugation of fluvialinate to an anilino acid.

explanation for some enterohepatic circulation of foreign compounds⁷.

We emphasize that these bile acid conjugates are not minor metabolites; collectively they represent 6 and 11% of the administered ¹⁴C dose in rats and chickens, respectively. Our suggestion that they have general occurrence is based largely on the promiscuous conjugation of an anilino acid with whatever bile acids predominate in the species investigated. Further studies should test the generality and toxicological ramifications of this excretion mechanism and may also identify previously unknown, intractable metabolic residues.

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Evidence for the lipidic nature of tight junction strands

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We examine here the proposition that membrane lipids^{1–4}, rather than intrinsic membrane proteins^{5–7}, are the principal structural elements of the strands comprising tight junctions. Our evidence, which is based on direct rapid freezing of newly formed tight junctions between rat prostate epithelial cells, indicates that individual tight junction strands are pairs of inverted cylindrical micelles sandwiched between linear fusions of the external membrane leaflets of adjacent cells. Although individual tight junction strands appear as continuous cylinders when fractured near the frozen surface, where ice crystals have not damaged the plasma membrane, they appear as rows of particles when fractured deeper in the frozen tissue. We now interpret these tight junction particles as remnants of intramembrane cylinders disrupted during freezing. The morphology and dimensions of the intact cylinders correspond to those of lipids in the cylindrical hexagonal II phase^{8,9} and this suggests that tight junction formation requires a phase transition of the planar lipid bilayer similar to that invoked in models of membrane fusion^{10,11}. Our morphological interpretation explains the known functional properties of tight junctions.

Tight junction strands have been assumed to consist of a row of intramembrane particles representing integral membrane proteins which on fixation (glutaraldehyde 1–5%) and cryoprotection (glycerol 20–30%) form the continuous fibrils observed in standard freeze-fracture replicas^{5–7,12–16}. This interpretation was based on the observation that a chain of particles replaces the continuous fibrils when the only preparative step is freezing by immersion in liquid Freon—a procedure which freezes tissue more slowly than current rapid freezing methods^{12,17}. Subsequent studies where mixtures of fibrils and particles were obtained after brief fixation in dilute glutaraldehyde were interpreted to confirm the proteinaceous nature

of tight junction strands⁷. Freeze-fracture has also been used to investigate whether the tight junction strand comprises a single fibril¹⁸ or a pair of fibrils, either lying side by side^{5,14} or, as most recently suggested, offset across the membrane contact⁶.

In the present experiments, fresh tissue was frozen directly with liquid helium in order to minimize the physical effects of ice crystal formation and to avoid the effects of chemical fixation and cryoprotection with glycerol (which may contain contaminants with potential cross-linking properties⁷). Pieces of ventral prostate gland were excised from adult Sprague–Dawley rats under light ether anaesthesia. The slices were incubated for 3–15 min in 0.1 M buffer, pH 7.4, at 37°C, placed on a rapid freezing stage and frozen with liquid helium by methods described elsewhere¹⁷. During this incubation, new tight junction strands form along the entire length of the lateral plasma membranes of adjacent epithelial cells³.

Near the original surface of the slice, where ice crystals do not impinge on the plasma membrane¹⁷, pairs of cylinders (9–11 nm in diameter) lie side by side along contacts between adjacent bilayers; each cylinder corresponds to a tight junction fibril. The cylinders form elongated segments that adhere to either the protoplasmic (P, Fig. 1a) or the exoplasmic (E, Fig. 1b) fracture face, leaving a complementary furrow on the other fracture face (Fig. 1a,c,d). Where the plane of fracture jumps from an E face of one membrane to the P face of the adjacent membrane two cylinders may be exposed (Fig. 1d) or one cylinder and a groove left by an adjacent cylinder that adhered to the complementary E face (Fig. 1c). For such planes of fracture to occur, the cylinders must be slightly offset with respect to each other⁶. This asymmetric disposition of the cylinders also explains why lateral views at junctional P face–P face transitions between abutting membranes of adjacent cells show only one of the cylinders, regardless of whether the transition is viewed from its apical or basal aspect (Fig. 1e). The diagram of a tight junction strand in Fig. 2a illustrates in cross-section the disposition of the two intramembrane cylinders. Figure 2b shows different observed planes of fracture through such a structure.

Deeper in the frozen tissue, where larger ice crystals impinge on the plasma membrane, cylinders are replaced by chains of particles that always appear on the E faces (Fig. 1f), but still leave continuous grooves on the P faces (Fig. 1g). Short segments of cylinder mixed with rows of particles are found over continuous grooves in both E (Fig. 1h) and P (Fig. 1i) faces of the plasma membrane at intermediate levels of freezing. The chains of particles seen on the E fracture faces of plasma membranes in poorly frozen areas (Fig. 1f) appear to be remnants of disrupted cylinders. Additional evidence that the tight junction strand is not a chain of particles is that a furrow, not a row of punctate depressions, is found on the complementary P face (Fig. 1g).

Although freeze-fracture images are normally analysed on the assumption that particles represent intramembrane proteins, it has recently been demonstrated that membranes made of lipids, which are capable of forming hexagonal II phase, have pits and particles on their freeze-fracture faces^{1,11,19–23}. Indeed, some published micrographs of protein-free liposomes suggest the presence of elongated cylinders within the lipid bilayers (see Fig. 1b in refs 23 and 24). The intramembrane cylinders at tight junctions have several features, such as hydrophobic environment (interior of the bilayer), morphology (smooth surface) and dimensions (9–11 nm diameter), which resemble those of inverted cylindrical lipid micelles (hexagonal II phase)^{8–10,25–27}, and which suggest that the cylinders found after direct rapid freezing could be differentiated lipidic domains rather than proteinaceous structures embedded in the membrane.

Other evidence that tight junction strands are lipidic, rather than chains of protein particles, is (1) cylinders obtained after glutaraldehyde fixation of pancreas or prostate tissues are disrupted after soaking in glycerol⁴ or acetone (B.K. and T.S.R., unpublished observations) but neither glycerol or acetone

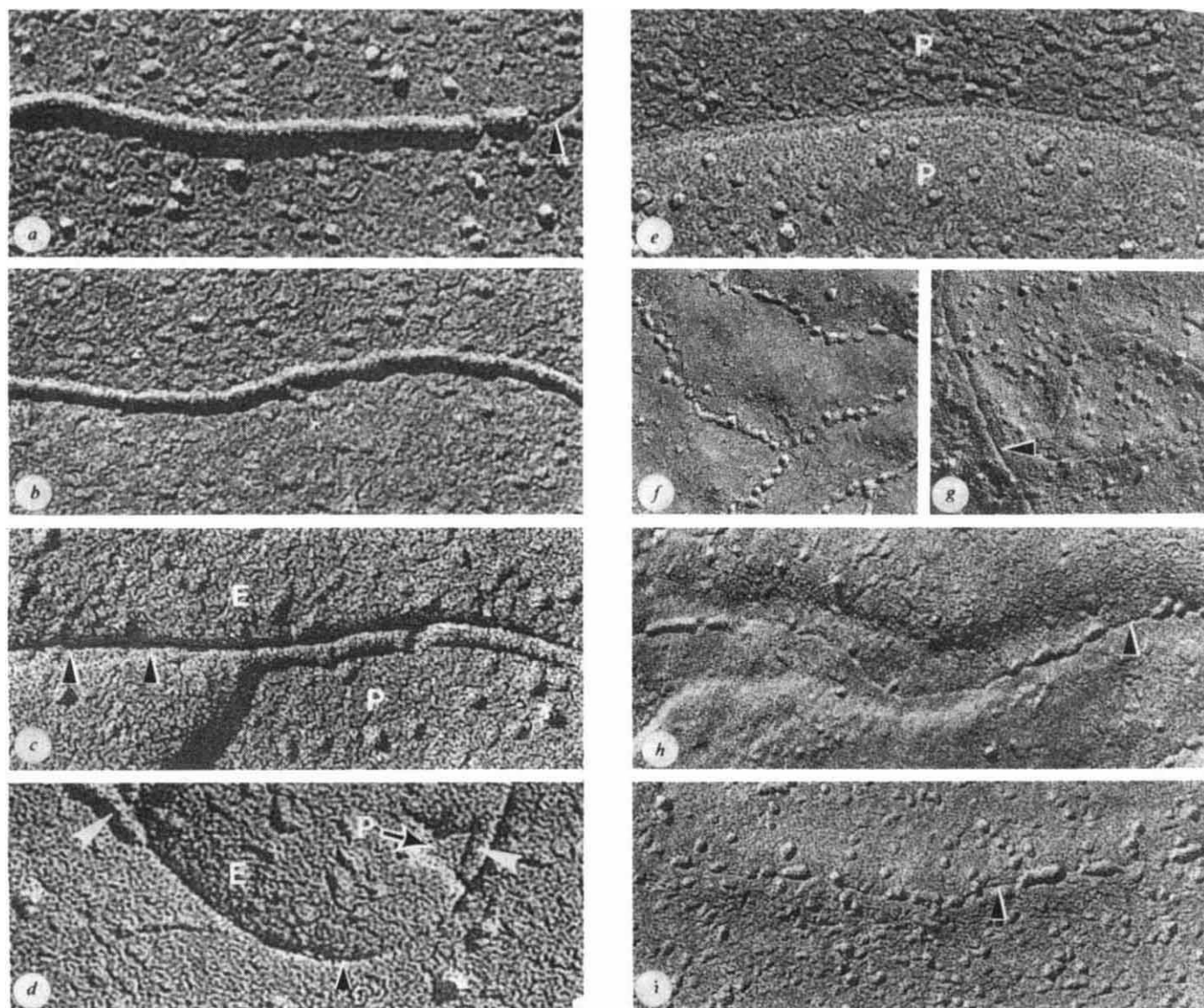


Fig. 1 Tight junction strands on fracture faces of plasma membranes of directly frozen rat ventral prostate epithelial cells. Continuous cylinders are found on P (a, c) and E (b) fracture faces near the original surface of the tissue where ice crystals have not visibly damaged the membranes. Two cylinders, one belonging to each of the pair of membranes participating in the tight junction, are seen in d (white arrowheads). Cylinders are also typically intact at junctional P face–P face transitions at abutting basal portions of plasma membranes of adjacent cells (e). At deeper regions in the specimen, where ice crystals impinge on membrane structure, chains of particles replace cylinders on E fracture faces (f), leaving a continuous groove on P faces (g). Mixtures of particles and short segments of cylinders are found over continuous grooves in both E (h) and P (i) faces from regions of intermediate ice crystal damage. P, P fracture faces; E, E fracture faces; $\blacktriangle$ point to continuous grooves. Platinum deposits are white. a–d, $\times 300,000$; e, $\times 250,000$; f, g, $\times 150,000$; h, i, $\times 170,000$.

would be expected to break covalent bonds formed by reaction of glutaraldehyde with integral membrane proteins. It is not even clear why glutaraldehyde fixation would affect the structure of the tight junction strand at all, unless it cross-links cytoplasmic²⁸ or peripheral membrane proteins associated with the tight junction strands. (2) Tight junction strands form within minutes in the absence of protein synthesis and without any noticeable change in the density or pattern of distribution of the intramembrane particles². However, punctate dimples and very short segments of tight junction strands are occasionally observed in plasma membranes of specimens rapidly frozen just as tight junction proliferation begins. We tentatively regard these dimples as early steps in tight junction formation because similar punctate dimples represent points of contact that become the sites of monolayer and bilayer rupture required for membrane fusion between artificial protein-free bilayers^{23,27}.

Transitions from planar bilayer to cylindrical micelles are also invoked in models of bilayer fusion^{10,11,21,27}. In these transi-

tions, the area of the polar side of the monolayer is thought to decrease while that of the hydrocarbon chain layer expands, so that the lipids pack into highly curved inverted cylindrical micelles with outward-facing hydrocarbon chains⁹. A similar process could lead to tight junction formation; segregation and packing of lipids during the phase transition would account for the formation of the intramembrane cylinder. These changes might involve a transition of the planar lipid bilayer to a cylindrical micelle in an hexagonal II phase, similar to that invoked in models of membrane fusion, but here somehow stabilized in the form of pairs of offset, inverted cylindrical micelles segregated within an interbilayer fusion. Figure 2c illustrates in cross-section the proposed micellar organization of the lipids at the tight junction strand. Figure 2d shows how this model can be applied to the interpretation of a freeze-fracture image.

The presence of a continuous cylinder intercalated between two protoplasmic faces as seen in apical or basal views of tight junctions (Fig. 1e) requires that there be a continuous, linear

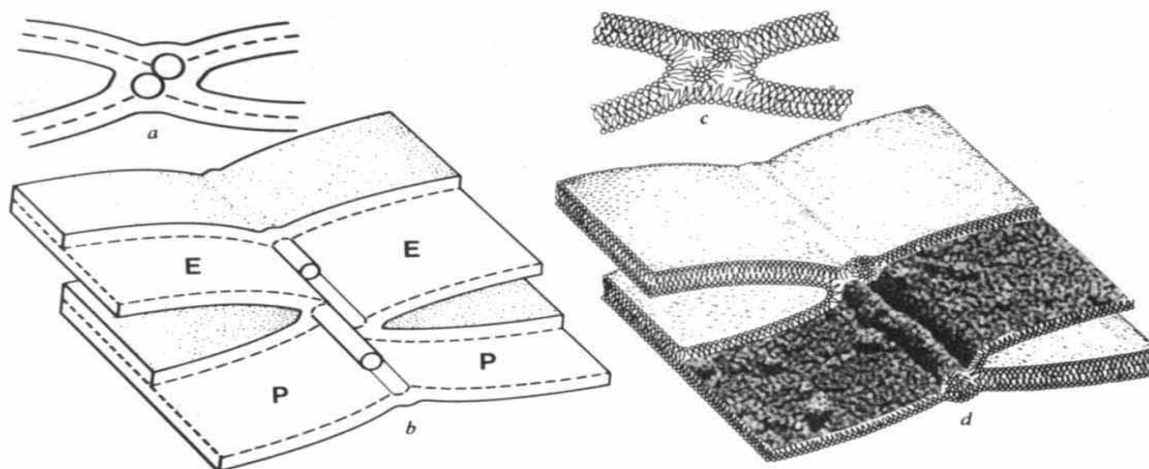


Fig. 2 *a*, Diagram of a cross-section of a tight junction strand illustrating the offset disposition of the pair of intramembrane cylinders. *b*, Diagram illustrating the paired offset cylinders at a tight junction strand and the different possible planes of fracture through such a junction. *c*, Proposed organization of the phospholipids at a tight junction strand. *d*, Diagram of phospholipids combined with freeze-fracture micrograph to show how fractures through lipid micelles could produce images characteristic of tight junctions.

interruption of the exoplasmic halves of the bilayer around an individual cell (Fig. 2*b*). However, the presence of inverted cylindrical micelles inside the membrane would also require continuity of the exoplasmic halves of bilayers of neighbouring cells across the tight junction strand (Fig. 2*c*). In fact, thin sections across tight junctions show points of partial membrane fusion, where only the exoplasmic leaflets of adjacent membranes have become continuous^{5,29,30}. Also, fluorescence quenching experiments show that lipid probes limited to the exoplasmic leaflets of cells joined by tight junctions are prevented from passing from their apical to their basal surface, while lipid probes that reach the protoplasmic leaflet can move freely around the cell circumference³¹. Thus the interpretation of tight junction structure presented here is consistent with their role as barriers that prevent diffusion between extracellular spaces and isolate apical and basal regions of the plasma membrane^{5,31}. It also shows how lipids and possibly other membrane components might flow from cell to cell within shared exoplasmic membrane leaflets.

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The structure of a pseudo intercalated complex between actinomycin and the DNA binding sequence d(GpC)

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Actinomycin D (AMD) is used clinically to treat tumours such as Wilms' tumour¹ and gestational choriocarcinoma². It inhibits transcription in most cellular systems^{3,4}, and binds to DNA, not to RNA^{3–5}, with a preference for guanine⁶. The study of the crystal structure of a 2:1 complex between deoxyguanosine and AMD demonstrated both stacking and hydrogen-bonding interactions between the drug and the guanine ring⁷. Solution studies^{8,9} have indicated that the drug binds preferentially to guanine–pyrimidine sequences, such as d(GpC), and that an intercalated complex forms with both DNA¹⁰ and DNA fragments¹¹. External binding¹² and intercalation^{10,13} models for the structure of the complex between AMD and DNA have been proposed, but until now no crystal structure of a complex between AMD and an oligonucleotide has been reported. As the smallest unit of DNA with the potential for forming an intercalated duplex is a self-complementary deoxydinucleoside monophosphate, we undertook the crystallographic analysis of the 2:1 complex between deoxyguanylyl-3', 5'-deoxycytidine, d(GpC), and AMD. The complex is found to form an unusual pseudo-intercalated structure.

Crystals of the complex were produced from aqueous solution with 3 mM d(GpC) and 1 mM AMD, using a vapour diffusion method. The red-orange crystals, some of which were thin hexagonal plates and others tetragonal rods, appeared after about 1 month. Spectroscopic analysis of dissolved crystals revealed a 2:1 ratio of d(GpC) to AMD. X-ray diffraction studies showed the hexagonal crystals to be isomorphous with the tetragonal ones and indicated a trigonal crystal system with cell dimensions $a = b = 16.577(3)$ Å and $c = 56.200(8)$ Å. The intensities were measured at 0°C on a diffractometer using 75 kV α radiation and a co-axial gas flow cooling system. Reflection intensities were measured in one octant (hkl) of reciprocal space with $2\theta \leq 62^\circ$ (1.5 Å resolution), using a $\theta/2\theta$ scan technique. A total of 3,706 reflections were collected and their intensities were corrected for absorption¹⁴ and decay. The data were then averaged assuming point group $\bar{3}m$ symmetry yielding 1,668 independent reflections. A systematic absence is observed in the 00 l zone with $l = 3n \pm 1$, thus the four possible space groups are P3₁21, P3₁12, P3₂21 and P3₂12. For the 1.5 Å resolution data the percentage of observed reflections on $1\sigma(\text{Fo}^2)$ and $2\sigma(\text{Fo}^2)$ levels are 74% and 58% respectively. From the observed crystal density (1.36 g cm⁻³) and unit cell volume (13,374.5 Å³), three complexes (molecular weight 2,366) and about 215 water molecules were predicted to be in the unit cell. All possible space groups require that the complex use the crystallographic 2-fold axis. AMD itself does not possess exact 2-fold symmetry, hence its amino and quinoid groups are statistically disordered.

The structure was determined by inspection of the Patterson map and computerized electron density fitting procedures¹⁵. Refinement procedures resolved the space group ambiguities. After several cycles of constrained-restrained least-squares refinement, water molecules began to appear in the difference Fourier maps and were added to the structure; most of them are disordered. The current R -factor is 19% for all data and 15% for 2σ data. The fractional coordinates of the complex have been deposited in the Cambridge data file.

The principal and surprising feature of the crystal structure (Figs 1a and 2a, b) is that it does not contain the intercalated duplex structure (Fig. 1b) proposed, for example, by Sobell and Jain¹³ on the basis of the crystal structure of the 2:1 complex between deoxyguanosine and AMD⁷. Instead the d(GpC) molecule is in the opened-out conformation with the phosphodiester torsion angles in the g^+g^+ range rather than the g^-g^- range found in self-complementary duplexes¹⁶⁻²¹; thus its conformation is similar to that displayed by ApU²² complexed with 9-amino acridine. The dinucleoside phosphate molecules are connected to each other via Watson-Crick base pairs, to form infinite chains, with each hydrogen-bonded d(GpC) chain making a right-handed spiral around the crystallographic screw axis. Each AMD molecule, whose structure is very similar to the one complexed with deoxyguanosine alone⁷, is located between these chains with its phenoxazone ring sandwiched between Watson-Crick base pairs from these different chains. The complex is thus pseudo-intercalated. In the AMD binding site, the phenoxazone ring stacks predominantly with both guanine rings and has very little overlap with the cytosines (Fig. 3). The guanine residues also interact with the cyclic peptide rings of AMD via hydrogen bonds between their amino group N-2 and ring nitrogen N-3 and the carbonyl oxygen and amide group of threonine residues respectively (Fig. 2). The same inter-complex hydrogen bonds were found in the 2:1 complex between deoxyguanosine and AMD⁷.

It is not yet clear why the d(GpC) does not form a duplex in the crystal because there is no chemical feature, such as base protonation, that would preclude such a structure. However, the rigid requirements for drug binding are incompatible with the conventional intercalated structures²¹, because the base pairs must form an unusual stacking pattern with the phenoxazone ring (Fig. 3) for the guanine to be positioned correctly for hydrogen bonding with the threonine (Fig. 2). Although we were able to build a stereochemically acceptable

intercalated model, the conformation of the dinucleoside phosphate backbone is unusual. This may explain in part the unusually large ³¹P-NMR downfield shifts observed in complexes between AMD and small oligomers²³⁻²⁵. These results also support the view that intercalation alone has a sequence preference at the dimer level. Although the crystal structures of both GpC¹⁶ and ApU¹⁷ show double-helical conformations, when ApU is complexed with 9-amino-acridine²² and when the deoxy form of GpC is complexed with AMD, duplexes do not form in the crystal. To date, without exception, all the known crystal structures of duplex-intercalated drug complexes have pyrimidine-3', 5'-purine sequences²¹ including the daunomycin hexamer complex²⁶. Furthermore, potential energy calculations for dinucleoside phosphates²⁷ indicate that standard intercalation geometries appear to favour that sequence.

This analysis supports the proposals^{10,12,13} that two 2-amino purines, such as guanine, are important, if not always essential²⁸, for the binding of AMD to DNA. The AMD molecule hydrogen-bonds only to guanine and not to cytosine, and it is the guanine base that is predominantly stacked with the phenoxazone ring (Fig. 3). Furthermore, only one of four possible orientations of the Watson-Crick G·C base pair allows hydrogen bonds to form between the cyclic peptide and the amino group N-2 and ring nitrogen N3 of the guanine ring. From considerations of base-drug interactions alone it would be predicted that AMD could bind to duplex d(GpC) but not to duplex d(CpG) because only the former sequence meets the hydrogen-bonding requirements. On the other hand, d(CpG) in an open conformation can complex with AMD⁸. In this structure the cytosine serves only as an accessory in anchoring the guanine in an optimal position for binding to AMD.

An examination of this structure shows why AMD cannot bind to RNA. If the deoxyribose of the guanosine (and not the cytidine) were changed to a ribose sugar, the O-2' hydroxyl group would have unusually short contacts with the amino group N-2 of the phenoxazone ring. This would also be the case in an intercalated model¹³ in which the distance between C-2' and the amino nitrogen N-2 is already quite short; it would thus be clearly impossible to replace the guanosine deoxyribose by ribose.

Using the results of this analysis it is possible to build a model of DNA in which guanine and cytosine residues from adjacent helices are involved in hydrogen-bonded cross-linking that is

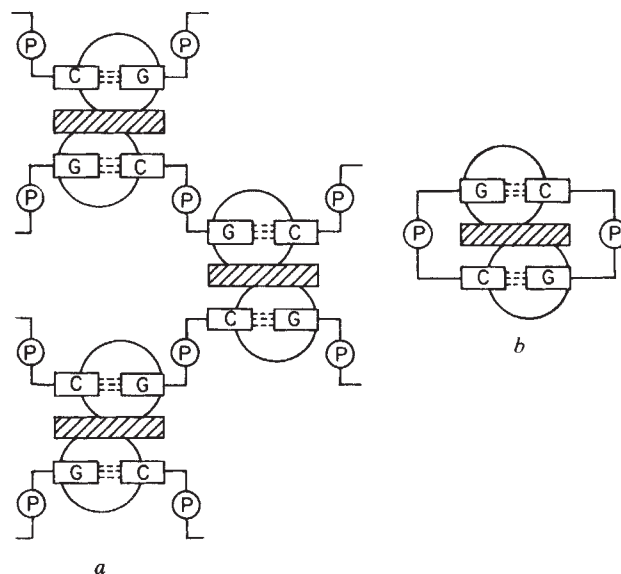


Fig. 1 a, Schematic view of the crystal structure of the 2:1 complex of d(GpC)-AMD. The phenoxazone rings are hatched and the cyclic pentapeptide rings appear as circles. $\boxed{G-C}$ indicates a d(GpC) molecule; --- indicates Watson-Crick base pair hydrogen bonds. b, Schematic view of an intercalated duplex model.

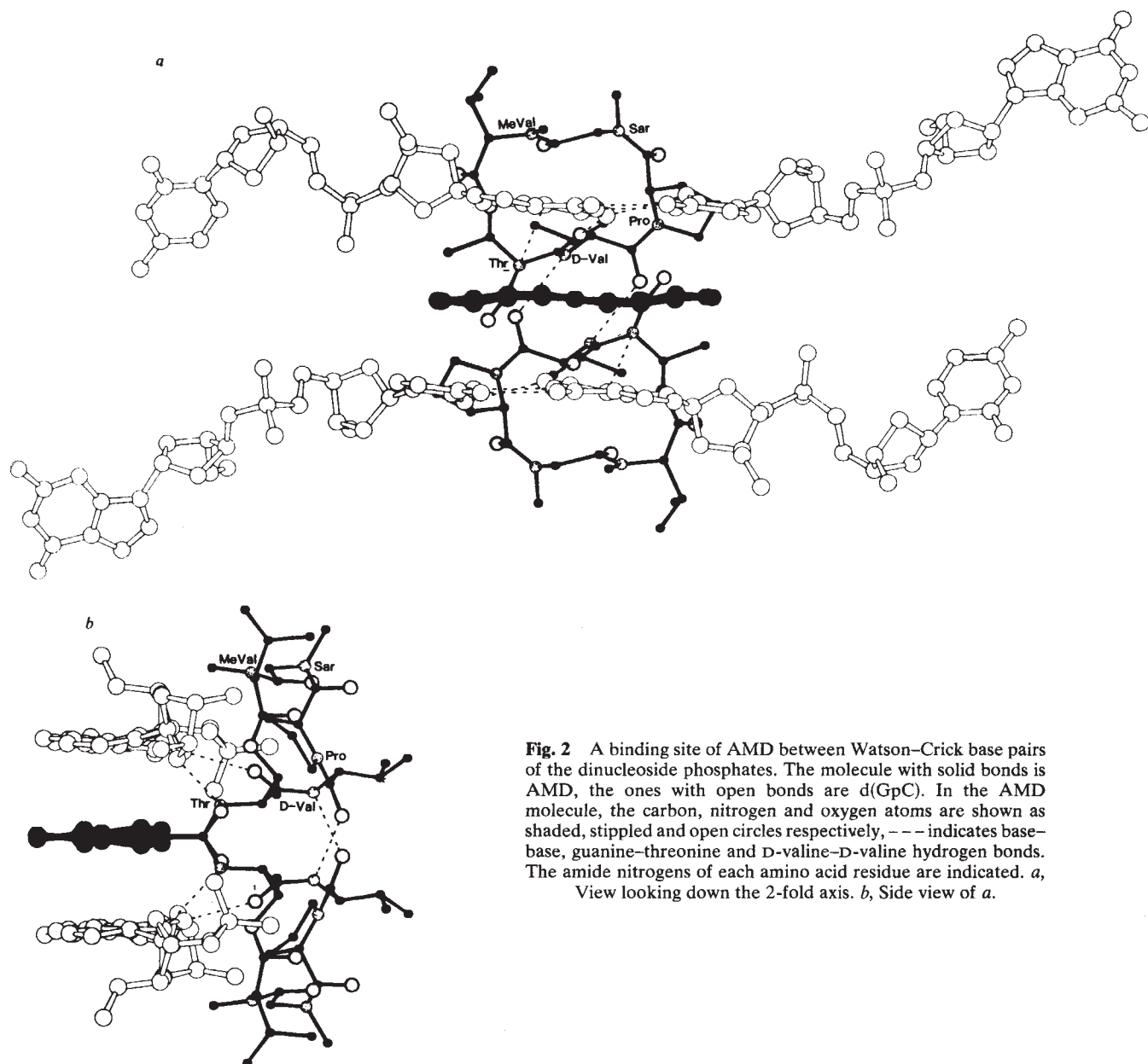
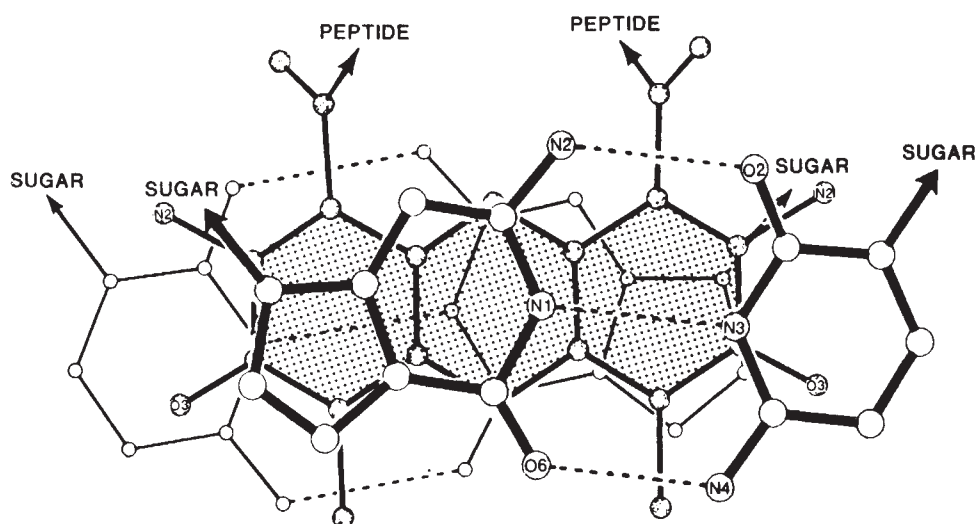


Fig. 3 View of the stacking of the planar ring systems. The phenoxazone ring is stippled; --- indicates Watson-Crick hydrogen bonds. Both the amino group (N-2) and the quinoid oxygen (O-3) of the phenoxazone ring are 2-fold disordered. Each atom thus has a half occupancy.



stabilized by AMD. Further studies are necessary to determine whether this type of structure can exist in oligo- or polynucleotide-AMD complexes and if such structures are biologically significant. In recent studies²⁹ of semisynthetic AMD derivatives where both amino N-2 and C-7 sites of the phenoxazone ring were modified by substituting bulky groups for hydrogen atoms, the analogues were found to be effective antitumour agents, to inhibit DNA and RNA synthesis and to bind to DNA. In these cases it is difficult to envision a sterically feasible intercalation model that also incorporates the peptide-base hydrogen bonds; however, it is easy to fit these semisynthetic AMDs into a cross-linked structure, suggesting an alternative

explanation for a part of AMD's chemotherapeutic action. Cross-linking may also provide an explanation for some of the unusual ³¹P-NMR results observed with short DNA oligomers in solution²³⁻²⁵. Clearly not all the activities of AMD need be explained by classical intercalation.

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A gene deletion ending at the midpoint of a repetitive DNA sequence in one form of hereditary persistence of fetal haemoglobin

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The form of hereditary persistence of fetal haemoglobin (HPFH) that commonly occurs in black populations is an inherited disorder of haemoglobin synthesis characterized by a uniformly high level of fetal haemoglobin (HbF) synthesis in all the erythroid cells of affected adult individuals^{1,2}. The precise molecular basis of the HPFH phenotype remains unknown, but is of great interest because of the knowledge that could be gained, through its understanding, of the mechanisms that regulate the expression of globin genes during development. The most common form of HPFH in black populations is associated with an extensive deletion that includes the normal adult (δ and β) globin genes and adjacent flanking DNA³⁻⁸. To investigate this disorder in more detail, we have cloned the DNA encompassing the region of the gene deletion in a case of HPFH and have determined the nucleotide sequence across the 5' end point of the deletion within the non- α -globin gene complex. We report here that this end point maps at the midpoint of a member of the 'AluI' family of repetitive sequences located approximately 4 kilobases (kb) to the 5' side of the δ -globin gene. Such repetitive sequences may be 'hot spots' of recombination, and are possibly involved in regulating gene expression.

The isolation and characterization of the HPFH DNA clone will be described elsewhere (D.T., S.M.W. and B.G.F., in preparation). The restriction endonuclease map of the human

non- α -globin gene cluster in the region of the HPFH deletion end point that was cloned is shown in Fig. 1. The deletion end point occurs ~4 kb 5' of the δ -globin gene. The normal DNA flanking the 5' extremity of δ -globin gene that was cloned by Lawn *et al.*⁹ was subjected to nucleotide sequence analysis in our laboratory and was shown to contain the following noteworthy structural features (see also Fig. 1): (1) an inverted pair of 300 nucleotide-long repetitive DNA sequences of the 'AluI' family (refs 10-14) separated by ~700 bp of intergenic DNA and located ~4 kb from the 5' end of the δ -globin gene, and (2) a remarkable segment of DNA located ~1 kb from the 5' end of the δ -globin gene that contains (with one exception) over 200 successive pyrimidine nucleotides on one strand of the DNA¹².

Figure 2 compares the nucleotide sequences of normal and HPFH DNA starting at the 5' *EcoRI* site of the 3.1-kb *EcoRI* DNA fragment adjacent and 5' to the 2.25-kb *EcoRI* DNA fragment bearing the 5' end of the δ -globin gene (Fig. 1; refs 9, 13). The two sequences show perfect homology with only two base differences as far as residue 370 from the 5' *EcoRI* site (Figure 2); beyond this point there is only very limited, patchy homology between the two DNA sequences. Figure 2 shows that the normal sequence retained in the HPFH patient extends half-way through the upstream (5') member of the pair of inverted *Alu* family DNA sequences located 5' to the δ -globin gene. Thus the deletion in HPFH removes >3,000 nucleotides of the DNA flanking the 5' end of the δ -globin gene, including one and a half members of the pair of inverted *Alu* family repetitive DNA sequences and an extensive polypyrimidine stretch, then extends through the δ - and β -globin genes for an unknown distance 3' of the β -globin gene. The 3' *Alu* family DNA sequence that is totally deleted in this case of HPFH is known to be transcribed *in vitro* by RNA polymerase III to yield an RNA molecule 490 nucleotides long (although it is unknown whether such transcription occurs *in vivo*), whereas the 5' *Alu* sequence that is half deleted is not transcribed *in vitro*^{10,11,14}; the transcriptional orientation of this 3' *Alu* sequence is the same as that of the δ -globin gene.

It has been postulated¹⁵ that the inter- γ - δ gene DNA may contain control elements, the deletion of which is responsible for the HPFH phenotype. Potential candidates for such regula-

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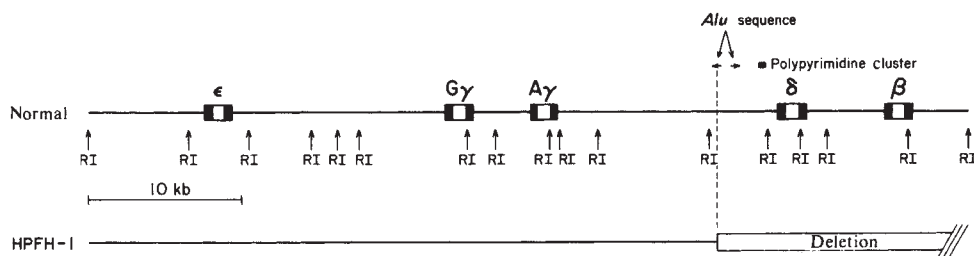


Fig. 1 Schematic representation of human non- α -globin gene cluster, showing the location of the deletion involved in the studied case of hereditary persistence of fetal haemoglobin (HPFH-1)^{4,6,7}. Vertical arrows indicate *EcoRI* restriction endonuclease cleavage sites. Solid boxes show coding regions, and open boxes the large intervening sequences of the globin genes. 'Alu sequence' indicates the two copies of moderately repetitive DNA sequences of the *Alu* family located 5' to the δ -globin gene.

tory sequences are the *Alu* repetitive DNA sequences and/or the polypyrimidine tract described above, although the specific molecular mechanisms by which deletion of these DNA elements could mediate failure of postnatal repression of the fetal γ -globin genes only on the *cis* chromosome bearing the deletion, are unknown and remain the subject of speculation. On the other hand, the coincidence of the deletion end point with an *Alu* family repetitive DNA sequence may indicate that *Alu* sequences, because of their number, chromosomal distribution and sequence homology, can serve as 'hot spots' for intra-chromosomal recombinational events that lead to DNA deletions. Such recombinational events would be expected to occur between two homologous *Alu* sequences and result in a fusion product containing portions of the two *Alu* sequences involved in the nonhomologous cross-over. In HPFH, however, only half of an *Alu* sequence persists as opposed to a full-length fused *Alu* sequence. One must therefore implicate other mechanisms for the loss of the other half of the expected fused *Alu* sequence. It is also possible that a cross-over occurred between two relatively nonhomologous DNA sequences on the

chromosome bearing the non α -globin genes. Only limited spotty homology has been previously noted for DNA sequences involved in various recombination events between simian virus 40 (SV40) viral DNA and host genomic DNA¹⁶.

Two other cases involving presumed recombination within *Alu* repetitive DNA sequences are known: one where a portion of an *Alu* repetitive DNA sequence from the genomic DNA of infected monkey kidney cells has been incorporated into SV40 (ref. 17) and another in which an α -globin gene has been partially deleted and fused to a repetitive DNA sequence of the *Alu* family¹⁸. In both cases, the recombinational events occurred internally within the *Alu* repetitive DNA sequences rather than at their boundary. It is not clear whether such occurrences are coincidental because of the high frequency of the *Alu* family sequences in the total genome, or whether there is some special propensity for nonhomologous recombination to occur within DNA stretches containing *Alu* repetitive sequences. Note, in this regard, that a number of deletions have been found in a yeast tRNA gene cluster that is flanked by short repetitive DNA sequences¹⁹.

Finally, because there is another deletion producing HPFH in blacks that has a different 5' end point extending across the same region of DNA^{7,8}, it seems unlikely that the HPFH phenotype in these cases is due to a second undetected mutation remote from the region of the deletion. It is possible, however, that the size of the deletion itself, the loss of downstream sequences, or the nature of the DNA brought as a result of the deletion into the vicinity of the fetal γ -globin genes is responsible in some way for the continuing expression of γ -globin genes in HPFH. Characterization of the DNA at the 3' end point of these HPFH deletions is in progress and may elucidate further the mechanisms responsible for the HPFH phenotype.

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481

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Fig. 2 Comparison of normal inter- γ - δ gene DNA sequence (upper line) with that of the DNA across the 5' end point of the deletion in HPFH (lower line). Asterisks show positions at which an identical nucleotide is found in both sequences. The short arrows overlying the sequence indicate the direct repeats that flank the extremities of the normal *Alu* DNA sequence and the solid line overlies the entire length of the normal *Alu* repetitive DNA sequence.

BOOK REVIEWS

The central problem in human evolution

C. Owen Lovejoy

DIET has always figured prominently in studies of the adaptation and evolution of mammals. This is to be expected, as most of the fossil record is composed of teeth and jaws, and feeding adaptations, aside from reproduction, are probably the most critical selective force in mammalian evolution. Moreover, the two-million-year archaeological record is made up of food remains and the tools used to obtain and process them. Superimpose upon this background such current debates as human sex roles, the quantitative analysis of archaeological sites and the forces at play in the origin of hominids, and the result is a cauldron of intense debate about facts and theories of almost every phase of human evolution.

The fourteen chapters comprising *Omnivorous Primates* are predominantly devoted directly to the problem of reconstructing the role of diet in human evolution. There are essentially four ways by which this can be done: by study of the archaeological record (three chapters); by study of the dietary habits of non-human primates (four chapters); by study of the dietary habits of contemporary hunter-gatherers (four chapters); and by direct study of the dental remains of hominids. Given the great advances and significant work which has been conducted in the past few years in the latter area, it is surprising that this volume contains no contributions dealing directly with these methods. One additional chapter which was not classified above deserves special mention. Alan Mann presents a thorough general essay on diet in the Plio-Pleistocene hominids using all of the four categories of data. He details dietary requirements of primates, reviews the historical development of various theories of feeding strategy in early human evolution (for example hunting, seed-eating, gathering), looks at the direct evidence available in cranial morphology, and summarizes with a discussion of models of early hominid dietary exploitation, concluding that the best model is one which posits a diet of marked diversity, significantly enhanced by the use of primitive tools with which to exploit underground food sources. It is of interest that the diet posited by Mann (as he himself points out) is quite similar to that found in Gombe chimpanzees (minus the underground rhizomes).

In his own paper Teleki provides a detailed account of this diet, including data on activity rhythms and feeding budgets. Most of his contribution, however, is

Omnivorous Primates: Gathering and Hunting in Human Evolution. Edited by Robert S. O. Harding and Geza Teleki. Pp.673. ISBN 0-231-04024-5. (Columbia University Press: 1981.) \$52, £28.60. *Woman The Gatherer.* Edited by Frances Dahlberg. Pp.250. ISBN 0-300-02572-6. (Yale University Press: 1981.) \$15, £10.50.

devoted to a factual description of chimpanzee meat consumption, in which he notes many aspects which may bear significantly on early human behaviour (for example evidence of "cooperative behaviour", food sharing and a sexual division of activity, to wit: "pursuit, capture and consumption of animals are primarily male activities, while collection of insects . . . is primarily a female activity"). In fact he concludes that "chimpanzees are ecologically, behaviourally, socially, and psychologically best suited to serve as a model for reconstructing human origins".

There are a number of similarities between Teleki's chimpanzees and the hunting behaviour in Gilgil baboons which is carefully presented by Shirley Strum (including cooperation, sexual differences in hunting behaviour and food sharing), but her contribution is of particular note for a different reason. Her continuous observation of a single group for over seven years has allowed her to chronicle significant changes in behaviour in response to ecological, demographic and social fluctuations. Her report strikes home the too-often forgotten fact that our knowledge of primate behaviour is based on but a few short-term studies of single groups which must be extended to millions of years of primate evolution.

As a whole, the volume is well-edited and produced, and will be valuable not only to those interested in human evolution and primate behaviour, but archaeological methods and ethnography as well. All of the contributions are competent and informative. Harding presents a general review of primate diet, and Hayden a similar world-wide review of diet in modern hunter-gatherers. Gould's review of food-acquisition and sharing behaviour in Australia and north-west California should prove particularly useful to prehistorians who must deal with the problem of the interpretation of food remains from archaeological sites. There is an informative up-date on the age-old

question of Pleistocene extinctions by David Webster.

In contrast to *Omnivorous Primates*, *Woman the Gatherer* contains virtually no contributions to the current debate. The title is an obvious word-play with respect to an earlier volume which has had far-reaching influence in modern anthropology, Lee and Devore's *Man the Hunter*. That volume was, in fact, more responsible than any other (despite some of its contributions and title to the contrary) in fully establishing the crucial role of women as gatherers and contributors to the success and function of pre-urban human societies. This new book, its title again to the contrary, contains little to amplify this now generally recognized fact. The article by McGrew, summarizing observations from a variety of field studies, is a thorough and highly competent account of (primarily female) chimpanzee behaviour, and in this regard is useful and informative. The model presented at the end of the article, however, falls short of actually being a model, but rather stands only as a brief account of a theoretical chimpanzee-like-hominoid to hominid transition without attention to selective mechanisms — the critical element in any successful model. A contribution by Zihlman, on the role of female gathering in human evolution, while again a useful account, suffers from the same deficiency.

Other papers in this volume, although of general interest, fall short of having a significant bearing on the actual role of females in long-term human evolution. Papers on hunting behaviour among Agta women, Mbuti womanhood, and the collection and preparation of food by Chipewyan women are useful contributions, but fall short of being directly applicable to the problems of human evolution. A long and involved paper by Catherine Berndt presents a rather cryptic account of the misuse of data obtained from observations of the Australian aborigines. Readers will suffer from her inherent assumption that they are intimately familiar with this arena of debate.

These two volumes, taken as a pair, raise certain rather interesting questions and paradoxes about viewpoints frequently adopted in the study of human evolution. The most disturbing of these is the failure to recognize the relatively enormous behavioural "gulf" between the australopithecines and modern man. Based upon what we know of the morphology of these

two taxa, that "gulf" must be approximately similar in magnitude to present differences between chimpanzees and hunter-gatherers. Primatologists (and one sees it frequently in these two volumes) often adopt an attitude that human behaviour is little more than slightly elaborated chimpanzee (or other primate) behaviour; ethnographers can, to the contrary, take the view that australopithecines were hunter-gatherers with robust faces and walnut-sized brains. Both groups would do well to study with great care the contributions to *Omnivorous Primates* by Freeman and Klein. These authors carefully document and chronicle the dramatic and progressive development of dietary shifts which took place in the middle and late Pleistocene in the Iberian Peninsula and the circumcoastal region of South Africa. While the question of the actual origin of hominids is critical and intriguing, the transformation of a

chimpanzee-like bipedal hominid into a cognitive, social and technological animal took place during the middle Pleistocene, a period for which no living analogues exist. There has been an overall assumption that *Homo erectus* was simply an intermediate between australopithecine and human being, but the archaeological evidence does much to contradict such an assumption. All living societies, no matter how "primitive", post-date the dramatic advances in upper Palaeolithic technology and the unquestionably equally dramatic alterations in social and subsistence behaviour which accompanied them. It is clear that we will lack a complete understanding of the process of human evolution until this vast yet crucial middle period is given the detailed attention it most sorely deserves. □

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certain words, for example "the" 10,144 times, "of" 7265, "in" 3904, "to" 3563; "you" which occurred three times was also suppressed.

To my amazement, Darwin did not include a single mention of the aardvark, and zoological appeared only twice with single entries for zoologist and zoologists. Geological, geologists and geology together merited 128 entries, thus emphasizing the relative importance of these two disciplines in Darwin's eyes. Even the creationists are well catered for in that they can readily list the number of qualifying prepositions, nouns, adverbs and adjectives used. Insights into Darwin's relationship with other scientists of the period can also be extracted from this work. Charles Lyell is mentioned 27 times in such phrases as "Lyell's noble views", "Lyell's grand work", "Lyell's profound remark", "Lyell's manual will bring home the truth". Huxley rates a mere 4 mentions, Murchison 4, Adam Sedgwick 2, whereas Owen and Agassiz with 18 and 10 respectively do much better.

The use of certain words must surely be significant: for example Darwin uses the first person singular some 999 times. And although Darwin's theory arose primarily from his circumnavigation of the world aboard the H.M.S. Beagle, this vessel is mentioned but twice, firstly in the opening sentence of the book: "When on board H.M.S. Beagle, as naturalist, I was much struck by certain" (there the entry ends). The other word that is remarkable for the circumspection with which it was used by Darwin is the last word in the book: "and most wonderful have been, and are being, evolved".

There is one further criticism that can be levelled at this volume: at no place is it possible to discover the full title of Darwin's book. Once again it is necessary to refer to the Harvard facsimile: *On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life*. □

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Anatomy of the *Origin of Species*

L. Beverly Halstead

A Concordance to Darwin's Origin of Species, 1st Edn. Edited by Paul H. Barrett, Donald J. Weinshank and Timothy T. Gottleber. Pp.834. ISBN 0-8014-1319-2. (Cornell University Press: 1982.) \$38.50, £27.

How appropriate it is, in the year marking one hundred years since Darwin's death, that his best-known book should join that other great British institution the "Complete Works of William Shakespeare" to say nothing of the Bible. All students of evolution and Charles Darwin will be indebted to Cornell University Press for their concordance to the *Origin of Species*, first edition.

This concordance is perhaps the supreme monument to what a computer can do with a book and also to what university teachers can accomplish with the help of undergraduates — who, in this case, patiently

typed 834 pages of text at 86 lines to a page. Unlike the concordance to the Bible, where the full context is listed under each entry and one can obtain all the useful quotations without ever having to open the Bible itself, with the present work such an approach is not possible. Each entry listed is printed in the centre of the page with sufficient of the adjacent words to fill a single line of print, no more and no less. This means that the entries rarely make up a complete sentence, and if they are at the end of a sentence the following quotation may well be entirely irrelevant to the entry. Reference to the first edition itself, or rather a facsimile, is thus essential. The publishers of the facsimile, Harvard University Press, should be duly grateful.

Every student of evolution will wish to possess this concordance, but it must be stressed that for all its thoroughness the three editors found it necessary to suppress

0411 groups under groups. This classification is EVIDENTLY not arbitrary like the grouping of the star
0201 a fair balance be struck between the good and EVIL caused by each part, each will be found on the
0490 and most wonderful have been, and are being, EVOLVED.
0009 nts have pollen utterly worthless, in the same EXACT condition as in the most sterile hybrids. We
0032 fs that this is not so in some cases, in which EXACT records have been kept; thus, to give a very

0297 heir species; and they do this the more readily IF the specimens come from different sub stages of
0297 which on my theory we ought to find. Moreover, IF we look to rather wider intervals, namely, to d
0300 gions of the whole world in organic beings; yet IF all the species were to be collected which ever
0301 nal gradations between any two or more species. IF such gradations were not fully preserved, trans
0301 enerally be local or confined to one place, but IF possessed of any decided advantage, or when fur
0301 logists, he ranked as new and distinct species. IF then, there be some degree of truth in these re
0301 eri and these links, let them be ever so close, IF found in different stages of the same formation
0302 to the belief in the transmutation of species. IF numerous species, belonging to the same genera
0303 succeeding formation such species will appear as IF suddenly created. I may here recall a remark fo
0304 If extracted from the chalk of Belgium. And, as IF to make the case as striking as possible, this
0305 north as they are at present. Even at this day, IF the Malay Archipelago were converted into land,
0307 actors in any degree intermediate between them. IF, moreover, they had been the progenitors of the
0307 umerous and improved descendants. Consequently, IF my theory be true, it is indisputable that befo
0307 lated before the Silurian epoch, is very great. IF these most ancient beds had been wholly worn aw
0309 ntervened during these enormously long periods. IF then we may infer anything from these facts, we
0309 nd. Nor should we be justified in assuming that IF, for instance, the bed of the Pacific Ocean wer
0312 cent beds, though undoubtedly of high antiquity IF measured by years, only one or two species are
0312 re, as far as we know, on the face of the earth. IF we may trust the observations of Philippi in Si
0313 seldom changed in exactly the same degree. Yet IF we compare any but the most closely related for

... the final word from Darwin.

... if a picture paints a thousand words ... , or here 19 examples of the 415 times "if" was used in the *Origin*.

Page ***** (Key Word) *****

Ion transport for enthusiasts

N. Michael Green

The Sarcoplasmic Reticulum: Transport and Energy Transduction. By Leopoldo de Meis. Pp.163. ISBN 0-471-05025-3. (Wiley:1981.) £29.25, \$52.50.

OVER the past 12 years the sarcoplasmic reticulum has become increasingly popular as a model system for the study of transport of ions across membranes. Physiology, electron microscopy, techniques of membrane reconstitution, lipid chemistry, protein chemistry and enzyme kinetics have all contributed to make this one of the best known of subcellular organelles.

This short monograph is concerned with the author's own field of kinetic studies on isolated vesicles of sarcoplasmic reticulum, to which he has made significant contributions. After two concise and informative introductory chapters, Dr de Meis restricts himself to this area. By concentrating his attention in this way he is able to present a variety of steady state and transient state kinetic experiments which have led to the generally accepted minimal cycle of at least eight reversible reactions that are responsible for Ca^{++} translocation. Since Ca^{++} , Mg^{++} and ATP each enter the cycle more than once, it has required considerable experimental ingenuity to provide even a qualitative description of the reaction sequence. The chief merit of the book is that it presents crucial experiments from many laboratories in well-illustrated detail, providing a valuable perspective for anyone trying to find his way through the 300 or more papers quoted. Mastery of the evidence presented will bring the reader to the end of 1980 and to a good position from which to cope with the arguments of 1981 and beyond, for the story is by no means over.

The author says very little on several important questions on which the evidence is conflicting and it would have been helpful to have had these problems more clearly delineated. They include the related questions of a monomeric versus a dimeric functional unit and of the number of Ca^{++} and ATP binding sites per peptide chain. The questions of co-transport or counter-transport of other ions and of the possible electrogenic nature of the pump are barely considered, while excitation-contraction coupling and Ca^{++} release mechanisms are explicitly excluded.

The final chapter on the role of water in the active site of the ATPase is rather speculative, being based in part on the effects on the stability of the acyl phosphate of dimethylsulphoxide, which is assumed to be a hydrophobic solvent; this assumption is hardly warranted by its very high dielectric constant. The remarkable changes in reactivity undergone by the acyl phosphate intermediate during the catalytic cycle are more likely to be a

consequence of local electronic influences than of a general change in accessibility of the catalytic site to water.

These are minor reservations, however, and do not seriously detract from the value of this book to all those interested in an analytical approach to ion transport. □

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Intention in mind

Richard Gregory

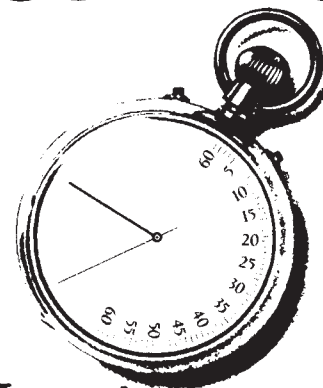
Mind Design: Philosophy, Psychology, Artificial Intelligence. Edited by John Haugeland. Pp.368. Hbk ISBN 0-226-08110-5; pbk ISBN 0-226-58052-1. (MIT Press: 1981.) Hbk \$21.50, £13.50; pbk \$10, £6.20. *Minds and Mechanisms: Philosophical Psychology and Computational Models.* By Margaret A. Boden. Pp.311. ISBN 0-855-27064-0. (Harvester/Cornell University Press: 1981.) £20, \$29.50.

Mind Design, a collection of 12 papers by computer scientists and philosophers of AI, was conceived by its editor, John Haugeland, as a sequel to A.R. Anderson's *Minds and Machines* which was published by Prentice-Hall in 1964. This new collection offers essays by Allen Newell and Herbert Simon; Zenon Pylyshyn; Marvin Minsky; Drew McDermott; Hubert Dreyfus; Hilary Putnam; Daniel Dennett; John Searle; Jerry Fodor; and Donald Davidson. There is also the particularly welcome essay, "Artificial Intelligence — A Personal View", by David Marr, whose recent death while at the height of his genius was a tragic blow for computer vision as well as for his many friends. The editor himself contributes an insightful Introduction, "Semantic Engines", and a rather less interesting essay on cognitivism.

In his Introduction, Haugeland asks how the psychology of thought can be properly scientific when thoughts cannot be observed; though the problem is not like that of galaxies or electrons, that they are too far away or too small. Referring to the "marvellously rich analogy with computers", and especially to a chess-playing machine, which "would be awkward to describe [in] its functioning by assigning geometrical shapes and locations to the internal program routines, yet has no immaterial soul" the crucial question is raised: how far is the human chess player like the machine that may beat him in intellectual combat?

A central issue throughout the book is

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Michael J. Ford,
Nature Conservancy Council

Choosing a wide range of natural examples, Dr Ford considers the different levels at which climatic effects operate on individual species and communities. Although the magnitude of climatic change this century is quite small, the author argues that its effect on natural flora and fauna may be disproportionate. Man's destruction of natural habitats includes climatically favourable areas providing refuge in times of climatic stress. If these disappear, certain species may be endangered.

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intention; here the American philosopher Dan Dennett has interesting things to say, also using the chess-playing computer example. He points out that even the designer cannot predict the next move of the machine from its design. He can only do so by considering rational and good moves of the game, which implies that the machine "chooses" rational and good moves. So, one is viewing machines such as these in intentional terms. Dennett, however, is careful to say that we may be right to attribute a lot more to human beings than this rather limited kind of intention, though he is not sure what this extra is.

Hubert Dreyfus is the respected devil's advocate of AI, whose book *What Computers Can't Do* (Harper & Row, 1972) provided, to the credit of all, reasoned criticisms that have been discussed with reason and not merely dismissed by the high priests of AI. Here Dreyfus adapts to the new developments of computer systems, starting with the universally acknowledged achievement of Winograd's natural language understanding program SHRDLU of 1972, which worked in a simplified micro-world, which was not much like our reality. Dreyfus objects to a common AI assumption, that such manageable micro-worlds can be generalized to the real world of physics and people. He suggests that proponents of AI are misled by the success of test-tube procedures in the natural sciences into thinking that these micro-worlds are even relevant; because although the test-tube worlds of physics obey physical laws, the computer micro-worlds may obey only their own laws. I suppose the answer is that "mental" laws are not physical laws anyway, so the whole exercise is outside physics; but it may still be science. Perhaps only physicists will strongly object to this conclusion.

Marvin Minsky invokes the notion of structures of knowledge — "frames" — to match common situations and to be adapted to the special features of situations. He attributes human intelligence to the individual's ability to select frames appropriately and rapidly. Selected knowledge frames are adapted to match the details of particular situations as they are encountered. This bears a resemblance to Bartlett's "Schemas" for locking together and giving significance to perceptions and memories. It is also related to David Marr's "Primal Sketch", his

initial descriptive stage of visual processing.

Several of the authors here express doubts on the scientific status of AI and its claims and results. For Marr,

a result in AI consists of the isolation of a particular information-processing problem, the formulation of a computational theory for it, the construction of an algorithm that implements it, and a practical demonstration that the algorithm is successful.

He points out, though, that there may be nice and neat successful solutions (which may not be found) and also messy solutions which may be as successful. Given the aesthetics of science, one may guess that AI will be accepted as properly scientific only if its solutions are both successful and elegant, whatever be the case for our own brain's functions — even when we are thinking about the challenging intellectual issues presented in these illuminating essays on artificial intelligence.

Margaret Boden's *Minds and Mechanisms* is a collection of her own papers on the philosophy of AI. Her concern with the concept of intention goes back to her first book, *Purposive Explanation in*

Psychology (Harvard University Press, 1972), and is also a theme of her widely acknowledged text, *Artificial Intelligence and Natural Man* (Harvester, 1977). Intention appears to be basic to any language system, and Professor Boden was largely responsible for pointing this out. Her strength lies in combining a deep knowledge of cognitive psychology, and what AI programs can and cannot at present do, with a background in both natural science and philosophy. The result is an authoritative book, though inevitably there is unevenness and some redundancy as the papers were written separately and for somewhat different audiences. Some of them take us more deeply into the issues of her previous books, and the technical detail presented here justifies careful reading for appreciating the fascination of mind struggling to be transmigrated into man-made machines that surprise and puzzle us. □

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Polymer dynamics revealed

F.A. Bovey

Molecular Motion in High Polymers. By R.T. Bailey, Alastair M. North and Richard A. Pethrick. Pp.415. ISBN 0-19-851333-X. (Clarendon/Oxford University Press: 1981.) £42.50, \$69.

ASTHEY state in their preface, in *Molecular Motion in High Polymers* the authors have essentially produced two books under one cover. Part A is primarily theoretical in nature and, after a general introduction, deals with normal vibrations and inelastic light scattering, various "hydrodynamic" models of chain motion and statistical theories of polymer molecular motion. The level of mathematical treatment is moderately high, and readers not comfortable with vector and tensor notation and matrix algebra will find themselves somewhat at sea.

Part B is described as a phenomenological treatment. It is more descriptive and the mathematical background is less prominent, although not at an elementary level. Most readers should be able to absorb this section with profit without necessarily resorting to Part A. Dielectric properties, photoluminescence, viscoelastic relaxation, ultrasonic relaxation, nuclear magnetic and electron spin resonance, spectroscopic and scattering phenomena, neutron scattering and diffusion are all well described.

My reservations about the book mainly concern the completeness of the treatment. More specifically, the literature citations

have a dated appearance. It should be possible to reference work within at most two years of the publication date, and in fact there is a reference to a 1980 paper by one of the authors. However, in general there are few references to papers later than 1975. This may be acceptable in an introductory textbook, but not in a scholarly treatise such as this. I was especially sensitive to this point in the chapter on nuclear magnetic and electron spin relaxation, my own field. Carbon-13 NMR, the area of greatest recent interest, is given rather short shrift. Although high-resolution carbon-13 NMR of solids is described, the treatment is very compressed. Carbon-13 relaxation in solution, a major area, occupies less than a page, and most of the relevant literature is omitted. It should also be pointed out that the section on the inversion-recovery method for measuring T_1 has been mislabelled "Carr-Purcell Experiments", a quite different technique used for measuring T_2 .

This selective treatment means that readers cannot count on being brought to the forefront of the topics described. Despite this reservation, I found the discussion informative and valuable. The book is well written and can be read with pleasure. □

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AI at MIT

At the end of April, MIT Press will publish a two-volume paperback edition of *Artificial Intelligence: An MIT Perspective* edited by Patrick Henry Winston and Richard Henry Brown. The original hardback edition was reviewed in *Nature* 282, 540; 1979. Price of the paperback version is \$12.50, £8.75 per volume; hardback is available at \$25, £17.50 per volume.

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Earth Sciences

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- VENGE, P. and LINDBOM, A. (eds). *The Inflammatory Process*. An Introduction to the Study of Cellular and Humoral Mechanisms. Pp.327. ISBN 91-22-00443-2. (Almqvist & Wiksell International, Stockholm, Sweden: 1981.) SwFr. 165.
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General

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ANNOUNCEMENTS

Awards

Professor George Weber (Laboratory for Experimental Oncology at Indiana University School of Medicine) has been elected for the 22nd Annual Clowes Award by the American Association for Cancer Research.

The Institution of Geologists has presented the Aberconway Medal to **Dr D.J. Burdon**.

Appointments

Dr David Mahoney is to head CSIRO's new Division of Tropical Animal Science.

Dr Robert E. Olson has been appointed associate dean for academic affairs at the University of Pittsburgh School of Medicine and also professor in the Departments of Biochemistry and Medicine.

James "Scotty" Miller has been named the first Director of Methods Development for the American Oil Chemists' Society.

Royal Society Fellows

At a meeting of the society on 18 March 1982, the following were elected Fellows of the Royal Society: **Dr Ulrich Wolfgang Arndt** (Medical Research Council Laboratory of Molecular Biology, Cambridge); **Professor Rodney James Baxter** (Theoretical Physics, Australian National University, Canberra); **Professor Michael Victor Berry** (Theoretical Physics, University of Bristol); **Professor James Derek Birchall** (ICI Ltd, Runcorn, Cheshire, and visiting professor of Materials Science, University of Surrey); **Professor Anthony David Bradshaw** (Botany, University of Liverpool); **Dr Daniel McGillivray Brown** (Organic Chemistry, University of Cambridge); **Dr Lawrence Michael Brown** (Physics, University of Cambridge); **Professor Bryan Campbell Clarke** (Genetics, University of Nottingham); **Professor William Maxwell Cowan** (Anatomy, Salk Institute for Biological Studies, San Diego, USA); **Professor Alan William Cuthbert** (Pharmacology, University of Cambridge); **Dr John Thomas Finch** (Medical Research Council Laboratory of Molecular Biology, Cambridge); **Professor Henry Edgar Hall** (Physics, University of Manchester); **Professor Michael Hart** (Physics, King's College, University of London); **Dr Eric John Hewitt** (Plant Physiology and Biochemistry Section of Long Ashton Research Station, Bristol, and Plant Physiology and Biochemistry, University of Bristol); **Professor Charles Antony Richard Hoare** (Computation, University of Oxford); **Professor Robert Francis Hudson** (Organic Chemistry, University of Kent at Canterbury); **Professor Kenneth Langstreth Johnson** (Engineering, University of Cambridge); **Professor William**

Johnson (Mechanics, University of Cambridge); **Professor Peter Julius Lachmann** (Tumour Immunology, University of Cambridge and Medical Research Council Unit on Mechanisms in Tumour Immunity, Cambridge); **Dr Ralph Lainson** (Wellcome Parasitology Unit, Belém, Brazil); **Dr Michael Francis Land** (Biological Sciences, University of Sussex); **Professor Peter John Lawrenson** (Electrical and Electronic Engineering, University of Leeds); **Professor William Russell Levick** (Physiology, John Curtin School of Medical Research, Australian National University, Canberra); **Professor Stephen Finney Mason** (Chemistry, King's College, University of London); **Dr Noreen Elizabeth Murray** (Molecular Biology, University of Edinburgh); **Sir Gustav Nossal** (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, and Medical Biology, University of Melbourne); **Professor Ronald Harry Ottewill** (Colloid Science, University of Bristol); **Dr William James Peacock** (Division of Plant Industry, CSIRO, Canberra, Australia); **Professor Philip James Edwin Peebles** (Physics, Princeton University, USA); **Peter Rainger** (British Broadcasting Corporation, London); **Professor Chintamani Nagesa Ramachandra Rao** (Indian Institute of Science, Bangalore); **Dr Lewis Edward John Roberts** (Atomic Energy Research Establishment, Harwell, and Board of the United Kingdom Atomic Energy Authority); **Professor George Stanley Rushbrooke** (Theoretical Physics, University of Newcastle upon Tyne); **Professor John George Sclater** (Marine Geophysics, Massachusetts Institute of Technology, USA); **Dr Graeme Bryce Segal** (Mathematics, University of Oxford); **Professor David Rostron Trentham** (Biochemistry and Biophysics, University of Pennsylvania, Philadelphia, USA); **Professor John Stewart Turner** (Geophysical Fluid Dynamics, Australian National University, Canberra); **Professor Thomas Gaskell Tutin** (Taxonomy, University of Leicester); **Professor John Conrad Waterlow** (Human Nutrition, London School of Hygiene and Tropical Medicine, University of London).

Meetings

16-18 May, **Biomedical Applications of LCEC and Voltammetry**, Indianapolis (LCEC Symposium, PO Box 2206, West Lafayette, Indianapolis 47906, USA).
17-18 May, **Cancer**, London (The Marie Curie Memorial Foundation, 124 Sloane St, London SW1, UK).
17-21 May, **Applied Combustion Technology**, Geneva (The Center for Professional Advancement, Postbus 19865, 1000 GW Amsterdam, The Netherlands).

18-20 May, **Roots at Work**, Wantage (Dr R.S. Bruce, ARC Letcombe Laboratory, Wantage, Oxon, UK).

19 May, **Landfall Leachate Symposium**, Oxford (Mr L. Evans, Education and Training Centre, Harwell Laboratory, Didcot, Oxon, UK).

24-27 May, **Ceramics Technology for the Non-Ceramist**, Amsterdam (The Centre for Professional Advancement, Postbus 19865, 1000 GW Amsterdam, The Netherlands).

24-26 May, **Male Transmission of Adverse Reproductive Effects**, Cincinnati (Marvin Legator, University of Texas, Galveston, Texas 77550, USA).

25-28 May, **Biological Basis of New Developments**, Minnesota (P. Rogers, 1060, Mayo Memorial Bldg, Box 196, 420 Delaware St, SE Minneapolis, Minnesota 55455, USA).

24-28 May, **Applied Pump Technology**, Amsterdam (The Center for Professional Advancement, Postbus 19865, 1000 GW Amsterdam, The Netherlands).

24-28 May, **CAGM Working Group on Weather and Animal Health**, Geneva (Research and Applications Programme Dept, World Meteorological Organization, Geneva, Switzerland).

25 May, **Chemistry which Imitates Biochemistry: Artificial Enzymes**, London (Miss J. Holman, The Ciba Foundation, 41 Portland Place, London W1, UK).

2-4 June, **International Chlorine Symposium**, London (The Conference Secretariat, Society of Chemical Industry, 14/15 Belgrave Square, London SW1, UK).

2-4 June, **Endocrine Lung in Health and Disease**, Washington DC (P. J. Petrossian, GWUMC-Office of CME, 2300 K. St, N.W. Washington DC 20037, USA).

13-16 June, **Birth Defects Conference**, Alabama (March of Dimes, 1275 Mamaroneck Ave, White Plains, New York 10605, USA).

29-June-1 July, **Crystallization Technology**, Amsterdam (The Center for Professional Advancement, Postbus 19865, 1000 GW Amsterdam, The Netherlands).

20 June-3 July, **The Application of Laser Light Scattering to the Study of Biological Motion**, Maratea (Charles Barker Lyons Ltd, 30 Farringdon St, London EC4, UK).

21-25 June, **Radioimmunoassay and Related Procedures in Medicine**, Vienna (IAEA, Wagramerstrasse 5, PO Box 100, A-1400 Vienna, Austria).

21 June-3 July, **Cancer Epidemiology**, Jabonna (International Agency for Research on Cancer, 150 cours Albert Thomas, F-69372 Lyon Cedex 08, France).

30 June-2 July, **Applications of Vibrational Spectroscopy**, Southampton (Dr J. Evans, Dept of Chemistry, The University, Southampton, UK).

5-9 July, **Mechanisms of Reactions in Solution**, Canterbury (Dr J. F. Gibson, The Royal Society of Chemistry, Burlington House, London W1, UK).

8-9 July, **Materials and Testing** London (The Institute of Physics, 47 Belgrave Square, London SW1, UK).

12-14 July, **Electronic Systems Effectiveness and Life Cycle Costing**, Norwich (Charles Baker Lyons Ltd, 30 Farringdon St, London EC4, UK).

17-25 July, **Microbiology**, Kansas (Dr Fung, Call Hall, Kansas State University, Manhattan, Kansas 66506, USA).

19-23 July, **Statistical Methods in Cancer Epidemiology**, Lyon (International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 8, France).

19-23 July, **Correlation Analysis in Organic Chemistry**, Hull (Dr J. F. Gibson, The Royal Society of Chemistry, Burlington House, London W1, UK).

19-24 July, **European Congress on Cell Biology**, Paris (1st European Congress on Cell Biology, 67, rue Maurice-Günsbourg, 94200 Ivry-sur Seine, France).

19-30 July, **Air-Sea Exchange of Gases and Particles**, Durham USA (Charles Barker Lyons Ltd, 30 Farringdon St, London EC4, UK).

27-29 July, **Strategies for Coping with Critical Issues Related to Materials and Minerals**, New Hampshire (FMS, 345 East 47th St, New York, New York 10017, USA).

26 July-6 August, **Genetic Engineering in Eukaryotes**, Pullman (Charles Barker Lyons Ltd, 30 Farringdon St, London EC4, UK).

31 July-6 August, **Living with Industry**, West Yorks, (The SISCON Summer School Secretary, Combined Studies in Science, University of Leeds, Leeds, UK).

1-11 August, **11th INQUA Congress**, Moscow (Prof. Dr Marton Pecs, Institute of Geography, Academy of Sciences, Budapest, Hungary).

2-5 August, **Meeting of the American Society for Virology**, Ithaca (H. S. Ginsberg, Dept of Microbiology, College of Physicians and Surgeons of Columbia University, 701 West 168th St, New York, New York 10032, USA).

2-6 August, **Applications of X-ray Analysis**, Denver (Mrs M. Cain, Denver Research Institute, University of Denver, Colorado 80208, USA).

5-6 August, **Computational Physics on the Distributed Array Processor**, Glasgow (The Institute of Physics, 47 Belgrave Square, London SW1, UK).

8-13 August, **9th Congress of the International Primatological Society**, Atlanta (Dr F. King, Yerkes Regional Primate Research Center, Emory University, Atlanta, Georgia 30322, USA).

9-13 August, **Joint Meeting, Electron Microscopy Society and Microbeam Analysis Society**, Washington DC (Prof. T.E. Mitchell, Dept of Metallurgy and Materials Science, Case Western Reserve University, Cleveland, Ohio 44106, USA).

15-17 August, **Functional Properties of Cereal Carbohydrates**, Winnipeg (Dr R.D. Hill, Plant Science, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2).

16-18 August, **Potential Utility of NMR in Research and Clinical Applications**, Boston (Dr M.R. Goldman, Internal Medicine, Massachusetts General Hospital, Boston, Massachusetts 02114, USA).

18-22 August, **SV40**, New York (G. Kist, Cold Spring Harbor Laboratory, PO Box 100, Cold Spring Harbor, New York 11724, USA).

15-19 August, **Genetic Engineering of Plants**, California (Tsune Kosuge, Dept of Plant Pathology, University of California, Davis, California 95616, USA).

16-29 August, **New Developments and Methods in Membrane Research and Biological Energy Transduction**, Spetsai (Prof., Dr K.W.A. Wirtz, State University of Utrecht, Laboratory of Biochemistry, NL-3508 TB, The Netherlands).

24-29 August, **Phage**, New York (G. Kist, Cold Spring Harbor Laboratory, PO Box 100, Cold Spring Harbor, New York 11724, USA).

30 August-2 September, **Planetary Rings**, Toulouse (CNES, Dept des Affaires Universitaires, 18, ave Edouard-Belin, 31055 Toulouse Cedex, France).

31 August-5 September, **Herpesvirus**, New York (G. Kist, Cold Spring Harbor Laboratory, New York 11724, USA).

31 August-2 September, **Very Large Baseline Interferometry Techniques**, Toulouse (CNES, Dept des Affaires Universitaires, 18, ave Edouard-Belin, 31055 Toulouse Cedex, France).

1-3 September, **Annual Symposium of the Uranium Institute**, London (The Uranium Institute, 8th Floor, New Zealand House, Haymarket, London SW1, UK).

1-3 September, **Environment and Safety Conference**, Wembley (Labmate Ltd, Newgate Sandpit Lane, St Albans, Herts, UK).

1-4 September, **Mutihormonal Cellular Regulations in Neuroendocrinology**, Colmar (Ph. Philippe Richard, Laboratoire de Physiologie Generale, Université Louis Pasteur, 21 rue René Descartes, 67084, Strasbourg Cedex, France).

6-10 September, **BA '82**, Liverpool (W. McQuarrie, University of Liverpool, Senate House, Abercromby Square, PO Box 147, Liverpool, UK).

8-9 September, **Vertebrate Palaeontology**, London (Geological Society, Burlington House, London W1, UK).

8-10 September, **Optics '82**, Edinburgh (Institute of Physics, 47 Belgrave Square, London SW1, UK).

8-15 September, **International Cancer Congress**, Seattle (Congress Operations Office, 4th and Blanchard Building, Suite 1800, Seattle, Washington 98121, USA).

8-10 September, **Coagulation, Cancer and Inflammation**, Virginia (Dr A. P. Ball, National Heart, Lung and Blood Institute,

Federal Building, Bethesda, Maryland 20205, USA).

8-18 September, **Principles and Methods in Receptor Binding**, Urbino (Dr I. Ceserani, Institute of Pharmacology and Pharmacognosy, University of Milan, Via A. del Sarto, 21, 20129 Milan, Italy).

9-10 September, **Volcanic Processes in Marginal Basins**, Keele (Geological Society of London, Burlington House, London W1, UK).

12-16 September, **Loss Prevention and Safety Promotion in the Process Industries**, Harrogate (The Institution of Chemical Engineers, George E. Davis Building, 165-171 Railway Terrace, Rugby, UK).

13-15 September, **Supercritical Fluids: Their Chemistry and Applications**, Cambridge (Dr W.R. Ladner, National Coal Board, Stoke Orchard, Cheltenham, Gloucester, UK).

13-16 September, **1982 Meteorological Society**, St Louis (Prof. Ghislaine Crozaz, Washington University, Box 1105, St Louis, Missouri 63130, USA).

13-17 September, **The Neutron and its Applications**, Cambridge (The Institute of Physics, 47 Belgrave Square, London SW1, UK).

13-17 September, **International Society for Photogrammetry and Remote Sensing**, Toulouse (GDTA, 18, ave Edouard-Belin, 31055 Toulouse Cedex, France).

13-17 September, **12th Congress of the International Federation of Societies of Cosmetic Chemists**, Paris (Societe Francaise de Cosmetologie, 44 rue du 22 Septembre, 92400 Courbevoie, France).

13-17 September, **World Filtration Congress**, Philadelphia (The Filtration Society, 2 Woodstock Rd, Croydon, Surrey, UK).

13-17 September, **Chromatography**, London (Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham, UK).

19-25 September, **Photosynthetic Prokaryotes**, Bordeaux (G. Stanier, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France).

14-16 September, **Neurophysics**, Cambridge (Dr G. Leng, ARC Institute of Animal Physiology, Babraham, Cambridge, UK).

15-17 September **1st Meeting of the British Liquid Crystal Group**, Southampton (Dr J.W. Emsley, Dept of Chemistry, The University, Southampton, UK).

20-24 September, **Carbon '82**, London (Society of Chemical Industry, 14/15 Belgrave Square, London SW1, UK).

20-24 September, **Antibiotics and Antibiosis in Agriculture**, Nottingham (Prof. M. Woodbine, University of Nottingham, Faculty of Agricultural Science, Sutton Bonington, Loughborough, Leicester, UK).

20-24 September, **10th Molecular Crystal Symposium**, Quebec (Dr D. Chartrand, National Research Council of Canada, Ottawa, Canada K1A 0R6).